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Short communication

Synthesis of structured phosphatidylcholine containing punicic acid by the lipase-catalyzed transesterification with pomegranate seed oil

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ABSTRACT

Incorporation of conjugated linolenic acid (punicic acid; 18:3, 9*c*,11*t*,13*c*) into egg-yolk phosphatidylcholine (PC) in the lipase-catalyzed transesterification process was the aim of this work. Pomegranate seed oil (PSO) containing over 77% of punicic acid was used as an acyl donor and three commercially available immobilized lipases were examined as biocatalysts. The effects of enzyme load, reaction time and molar ratio of substrates (PC:PSO) on the incorporation of punicic acid into the modified PC were tested. In all experiments the best results were obtained using lipase B from *Candida antarctica*. In optimum conditions (20% of enzyme load; 1:3 PC:PSO molar ratio; 72 h), the incorporation of punicic acid into the *sn*-1 position of PC was 56%. Additionally, the total content of polyunsaturated fatty acids in modified PC was almost 50%.

1. Introduction

Conjugated fatty acids (CFAs) are a mixture of positional and geometric isomers of polyunsaturated fatty acids (PUFAs) with conjugated double bonds. CFAs can be produced commercially by alkaline isomerization of PUFA-rich oils [1]. However, some of them occur in nature. Conjugated linoleic acid (CLA, predominantly the 9*c*,11*t* isomer), has been detected in food products from ruminants [2]. Conjugated trienoic fatty acids are found in some plant seed oils; for example punicic acid (9*c*,11*t*,13*c*-conjugated linolenic acid (CLNA)) in pomegranate (*Punica granatum*) seed oil, α-eleostearic acid (9*c*,11*t*,13*t*-CLNA) in bitter gourd (*Momordica charantia*) oil, catalpic acid (9*t*,11*t*,13*c*-CLNA) in catalpa (*Catalpa ovata*) seed oil, calendic acid (8*t*,10*t*,12*c*-CLNA) in pot marigold (*Calendula officinalis*) seed oil and jacaric acid (8*c*,10*t*,12*c*) in jacaranda (*Jacaranda* sp.) oil. They make up 72%, 60–70%, 31%, 33% and 32% of the total fatty acid in these oils, respectively [3].

CLA has been extensively studied for its beneficial biological activities in the improvement of immune function and prevention of atherosclerosis, cancer, obesity and hypertension [4]. Various CLNAs have also exhibited interesting biological effects such as: cancer cell killing activity *in vitro* [5–7], improving immune function and lipid metabolism [8], prevention and treatment of inflammation [9], decreasing body fat content [10] or hypoglycemic, hypolipidemic [11] and antioxidant properties [12]. Some studies indicate that CLNAs have stronger anticarcinogenic activities than conjugated dienoic fatty acids [13].

Phospholipids (PLs) are another class of lipids with many proven biological functions. They play a crucial role in membrane integrity, permeability and fluidity. PLs have been shown to reduce the levels of cholesterol and triglycerides in blood, prevent neurological diseases, and repair damaged liver tissue [14]. Due to their amphipathic character, PLs have been widely applied in food, cosmetic and pharmaceutical products as natural emulsifiers and components of liposomes [15].

The beneficial effect of the application of CFAs in food and pharmaceutical products as free fatty acids is limited. Phospholipids are a particularly effective and versatile system for the delivery of bioactive fatty acids, because they increase their bioavailability [16].

Enzymatic modification of phospholipids is conducted using lipases or phospholipases to remove or replace the acyl chains at position *sn*-1 or *sn*-2 of PL. Considerable efforts are being made mostly to incorporate health-promoting fatty acids from the *n*-3 or *n*-6 family [17–19]. Only one report has been published so far on the enzymatic incorporation of CLNA into PL [20]. However, a number of studies on the incorporation of CLA into triacylglycerols and phospholipids have been reported and in most cases processes of enzymatic acidolysis of PL have been applied [16,21–23]. Searching for natural sources of fatty acids as the acyl donors applied to the production of structured phospholipids in this

Abbreviations: PC, phosphatidylcholine; PSO, pomegranate seed oil; CFA, conjugated fatty acid; PUFA, polyunsaturated fatty acid; CLA, conjugated linoleic acid; CLNA, conjugated linolenic acid; PL, phospholipids; ALA, α-linolenic acid; LPC, lysophosphatidylcholine; TAG, triacylglycerol; TLC, thin-layer chromatography; FA, fatty acid; FAME, fatty acid methyl esters; FAEE, fatty acid ethyl esters; GC, gas chromatography; CAL-B, *Candida antarctica* lipase B.

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paper we propose a different reaction system — enzyme-catalyzed interesterification of egg yolk-PC with pomegranate seed oil (PSO). During this reaction the acids from the *sn*-1 position of PC are exchanged to those occurring in the *sn*-1 and *sn*-3 positions of triacylglycerols (TAGs) from oils. Such enzymatic reaction system excludes the use of phospholipases and requires 1,3-regioselective lipases which are active towards both starting phospholipids and triacylglycerols used as the acyl donors. This approach was successfully applied previously to produce ALA-, linoleic- and γ-linolenic-enriched PC in the lipase-catalyzed interesterification of egg-yolk PC with different plant oils [24].

In the presented work the effect of enzyme load, reaction time and molar ratio of substrates on the incorporation of punicic acid from PSO in the modified PC was studied.

2. Experimental

2.1. Materials and chemicals

Lohmann Brown hens' eggs were a gift from the Tronina factory. Pomegranate seed oil (PSO) was purchased from the ETJA Co., Poland. Lipozyme TL IM (a silica granulated *Thermomyces lanuginosus* lipase preparation, 250 U/g), was a gift from the Novozymes A/S (Bagsvaerd, Denmark). Lipase B from *Candida antarctica* (synonym: Novozym 435, >5000 U/g), and lipase from *Mucor miehei* (Lipozyme®, >30 U/g), were purchased from Sigma-Aldrich (St. Louis, MO, USA). A boron trifluoride methanol complex solution (13–15% BF_3 × MeOH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Boron trifluoride ethyl etherate (48% BF_3/Et_2O) was bought from Fluka. Silica gel-coated aluminum plates (Kieselgel 60 F_{254}, 0.2 mm) used in thin layer chromatography (TLC) and the silica gel (Kieselgel 60, 230–400 mesh) used in the column chromatography were purchased from Merck.

2.2. Isolation of PC from egg yolk

A pure phospholipid fraction (1.35 g) was isolated from 20 g of the fresh egg-yolk according to the method described by Gładkowski et al. [25]. The pure PC (0.9 g) was separated from this fraction by silica gel column chromatography (chloroform/methanol/water, 65:25:4, *v*/*v*/*v*).

2.3. The lipase-catalyzed interesterifiction of PC with pomegranate seed oil

The egg-yolk PC (0.13 mmol, 100 mg) was mixed with PSO (at different molar ratios of substrates 1:2, 1:3 and 1:5 PC/PSO) in 5 mL of hexane and then 70 mg of lipase (20% by weight of substrates) was added. The reactions were carried out using three different lipases, in N_2 atmosphere, at 50 °C. At 1:3 PC\PSO molar ratio the effect of enzyme load (10, 20, 30 and 40%) was tested in another set of experiments for two enzymes with high activity. The reaction mixtures were agitated in a magnetic stirrer at 300 rpm and stopped at the selected time intervals by enzyme filtration. Modified PC and lysophosphatidylcholine (LPC) were separated from the mixtures by column chromatography. All experiments were carried out in triplicates. Results in Table 1 and Figs. 1–3 are reported as means of triplicate experiments ± standard deviations (SD).

2.4. Analysis of substrates and products

Purity of modified PC was analyzed by TLC on silica gel-coated aluminum plates (chloroform/methanol/water, 65:25:4, *v*/*v*/*v*).

Fatty acid profiles of starting materials and products were determined after their conversion to the corresponding fatty acid methyl esters (FAME) according to the following procedure: samples (50 mg) were dissolved in 2 mL of 0.5 M methanolic NaOH solution. After heating under reflux (2 min) 1 mL of BF_3 × MeOH complex solution was added and the mixtures were heated for 3 min and cooled. Products were extracted with 1 mL of hexane and the organic layers were washed with a saturated NaCl solution. Hexane extracts were dried and analyzed directly by gas chromatography (GC) on an Agilent 6890N instrument (Agilent Technologies, Santa Clara, CA, US) equipped with a 70% cyanopropyl polysilphenylene-siloxane column (TR FAME, 30 m length, 0.25 mm diameter, 0.25 μm film thickness). The oven temperature was first set at 160 °C for 3 min and then raised to 220 °C (rate 5 °C min^{-1}) and next to 260 °C at 30 °C min^{-1} and held there for 3 min. The injector and flame ionization detector temperatures were 250 °C and 280 °C, respectively. The FAME were identified by comparing their retention times with those of a standard FAME mixture (Supelco 37 FAME Mix) purchased from Sigma-Aldrich.

2.5. Positional analysis of fatty acids in native and modified PC

The procedure was based on regiospecific Lipozyme®-catalyzed ethanolysis of PC. The details of the procedure were described in our previous paper [26].

3. Results and discussion

Our studies were aimed at the synthesis of structured phospholipids with punicic acid (9*c*,11*t*,13*c*-CLNA) using natural substrates and enzymes. The starting material was PC isolated from the Lohmann Brown hen's eggs and pomegranate seed oil (PSO) containing FAs in the form of triacylglycerols (TAGs) which were used as the acyl donor. Both of them were previously subjected to GC analysis to determine fatty acid composition. The results showed (Table 1) that the predominating FAs in PC were palmitic acid (16:0, 38%) and oleic acid (18:1, 30%). The former as well as another saturated stearic acid (18:0, 15%) is located in the *sn*-1 position (over 90%) whereas the *sn*-2 position is occupied by unsaturated acids (95%). FAs from the *n*-6 family are represented by linoleic acid (18:2, 11%) and arachidonic acid (20:4, 3%)

Table 1
Total and positional fatty acid composition (% according to GC) of native egg-yolk PC and CLNA-enriched phosphatidylcholine (CLNA-PC) obtained after Novozym 435 and Lipozyme®-catalyzed interesterification of egg-yolk PC with PSO[a].

FA	Native egg-yolk PC			CLNA-enriched PC					
				CAL-B			Lipozyme®		
	Total	*sn*-1	*sn*-2	Total	*sn*-1	*sn*-2	Total	*sn*-1	*sn*-2
C16:0	38 ± 2	63 ± 2.5	3 ± 0.5	10 ± 1.5	18 ± 1.5	3 ± 0.5	27 ± 1.5	45 ± 3	4 ± 0.5
C16:1	2 ± 0.5	2 ± 0.4	2 ± 0.5	1 ± 0.3	2 ± 0.3	–	1 ± 0.5	1 ± 0.2	–
C18:0	15 ± 1	29 ± 1.5	–	6 ± 1	10 ± 1	2 ± 0.4	11 ± 2	20 ± 2	–
C18:1	30 ± 1.5	5 ± 0.7	57 ± 1.5	34 ± 2	9 ± 1	49 ± 3	31 ± 2.5	1 ± 0.2	54 ± 2
C18:2	11 ± 0.8	1 ± 0.3	28 ± 1	15 ± 1.5	4 ± 0.5	31 ± 2	12 ± 1.5	1 ± 0.3	35 ± 2
C18:3 (punicic acid)	–	–	–	**29** ± 2	**56** ± 2	**5** ± 1	**15** ± 1	**32** ± 2	**1** ± 0.5
C20:4	3 ± 0.5	–	7 ± 1	3 ± 0.6	1 ± 0.2	7 ± 1	3 ± 0.5	–	6 ± 1
C22:6	1 ± 0.3	–	3 ± 0.7	2 ± 0.5	–	3 ± 0.5	–	–	–

[a] The values presented are means of triplicate experiments ± SD. Reaction conditions: enzyme load 20% (*w*/*w*), temperature 50 °C, 1:3 PC/PSO molar ratio, reaction time 72 h).