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Lipase-catalyzed enrichment of egg yolk phosphatidylcholine with conjugated linoleic acid

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ABSTRACT

The investigations were carried out to elaborate an efficient process of lipase-catalyzed acidolysis of egg yolk phosphatidylcholine (PC) with conjugated linoleic acid (CLA) taking into account both a high degree of incorporation into phospholipids (PLs) as well as the high yields of modified PLs. Therefore a term of "effective incorporation of CLA" (E_{CLA}) was proposed as the most complex criterion for the evaluation of effectiveness of the process. The best results were achieved when Lipozyme RM IM was applied and the reaction conditions were as follows: CLA/PC molar ratio 8:1; enzyme loading 24% wt. based on substrates; reaction medium heptane; temperature 45 °C; time 36 h. Reaction was conducted under initial $a_w = 0.33$ which was changed after 12 h to $a_w = 0.11$. Under these conditions incorporation of CLA (calculated as % of total FA) into modified PC and lysophosphatidylcholine (LPC) reached 33.8% and 50.1%, respectively. The E_{CLA} incorporation reached 39.0%. The yields of PC and LPC were 39.5% and 25.3%, respectively. The results showed the highest effective incorporation of CLA into PC in comparison to the data reported previously.

1. Introduction

Natural conjugated linoleic acid (CLA) as a mixture of positional and geometric isomers occurs mainly in products derived from poligastric animals: cattle, sheep and goats (0.6–1.2 mg CLA/g fat) [1,2]. The most common isomer in dairy products is *cis*-9,*trans*-11 CLA, which represents 80–90% of all CLA isomers [3–5]. It is the result of biohydrogenation processes occurring in rumen and conducted by rumen bacteria, mainly *Butyrivibrio fibrisolvens* [6,7]. Vegetable products, fish, seafood and meat from monogastric animals contain a low levels of CLA, ranging from 0.1 mg to 0.7 mg CLA/g fat [3].

Various biological properties of CLA have been reported in the literature. Anticancerogenic activities of CLA isomers toward human tumor cell lines and in rodent models have been studied [8–11]. Moreover, it has been reported that CLA reduces atherosclerosis risk [12,13] and stimulates immune system in mice [14]. Isomer *trans*-10,*cis*-12 contributes to the reduction of body fat level [15] whereas *cis*-9,*trans*-11 isomer enhances body weight gain and feed efficiency in animals [16]. Recent reviews of McCrorie et al. [17] and Dilzer and Park [18] showed that some of the human studies also confirmed the health-promoting effects of CLA, which can be achieved by diet supplementation. There are two general methods for food fortification with CLA. The first one uses either appropriate feeds to increase the CLA content in meat and milk of animals or bacterial strains capable to produce CLA in fermented dairy product. In this method enrichment of fish and sea food [19], pork [20] and dairy products [21–23] was achieved. The second approach is direct addition of CLA preparations to the food products during the manufacturing process.

A number of dietary supplements containing mainly a mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers or their esters are industrially produced. Formulations containing phospholipids (PLs) as a CLA carriers are not commercially available. This form of CLA preparation can be delivered more effectively because of better

Abbreviations: ANL, lipase from *Aspergillus niger*; ARA, arachidonic acid; BCL, lipase from *Burkholderia cepacia*; CALB, lipase from *Candida antarctica* B; CLA, conjugated linoleic acid; CRL, lipase from *Candida rugose*; E_{CLA}, effective incorporation of CLA; GPC, *sn*-glicero-3-phosphocholine; HPL, lipase from hog pancreas; LPC_{CLA}, percentage incorporation of CLA into modified lysophosphatidylcholine; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; PC-CLA, phosphatidylcholine with CLA residue; PC_{CLA}, percentage incorporation into *sn*-1 position of modified phosphatidylcholine; POA, palmitoleic acid; PL, phospholipid; PUFA, polyunsaturated fatty acid; RML, lipase from *Rhizomucor miehei*; SA, stearic acid; TLL, lipase from *Thermomyces lanuginosus*.

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absorption from digestive tract due to the emulsifying properties of phospholipid.

In this paper a comprehensive study on the lipase-catalyzed acidolysis of phosphatidylcholine from egg yolk with mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of conjugated linoleic acid is presented. The influence of many factors *i.e.*, temperature, solvent, substrate molar ratio, enzyme dosage and water activity on the incorporation degree and yield of products has been evaluated. The usage of term "effective incorporation of CLA" which is understood as the number of moles of CLA incorporated into the phospholipid molecules in relation to the initial amount of phosphatidylcholine substrate is proposed as a main parameter in optimization of reaction conditions.

2. Materials and methods

2.1. Chemicals and enzymes

Conjugated linoleic acid (purity: 99%) was prepared according to the method described previously [24]. Isomeric composition of CLA were as follows: *c*9,*t*11, 44.1%; *t*10,*c*12, 42.6%; all-*c*,*c* 3.3%; all-*t*,*t* 8.3%; other, 1.7%. L-α-phosphatidylcholine (PC) (≥99%) and L-α-lysophosphatidylcholine (LPC) (≥99%) from egg yolk were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were used as HPLC and TLC standards. 1,2-Di(conjugated) linoleoyl-*sn*-glycero-3-phosphocholine and 1-(conjugated) linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine were synthesized according to the method described previously [25,26] and were used as the HPLC standards.

Immobilized lipase from *Thermomyces lanuginosus* (Lipozyme TL IM, 250 U/g) was supplied by Novozymes A/S (Bagsvaerd, Danemark). Lipase B from *Candida antarctica* immobilized on acrylic resin (Novozym 435, >10,000 U/g) and lyophilized lipase from *Aspergillus niger* (200 U/g) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lipase from *Burkholderia cepacia* (Amano PS, 40 U/mg) was provided by Aldrich Chemical Co. (Milwaukee, WI, USA). Immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM, >30 U/g), lyophilized lipase from hog pancreas (27.4 U/mg) and lipase from *Candida rugosa* (2.8 U/mg) were purchased from Fluka (Buchs, Switzerland). All organic solvents and chemicals were of analytical grade and were supplied by Sigma–Aldrich (St. Louis, MO, USA). The TLC pre-coated silica gel 60 F_{254} plates, silica gel (Kieselgel 60, 230–400 mesh) used in column chromatography and all HPLC grade solvents (Merck LiChrosolv® Reag.) used in liquid chromatography were purchased from Merck (Darmstadt, Germany).

2.2. General methods

2.2.1. TLC analysis

Phospholipids were analyzed using the mixture of $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v) as a developing system. Compounds were detected using a solution of cerium sulphate (10 g) and phosphoromolibdenic acid (20 g) in 1 L of 10% H_2SO_4 followed by heating [25].

2.2.2. GC analysis

The fatty acid methyl esters (FAMEs) were analyzed by gas chromatography (GC) on an Agilent 6890N apparatus with a flame ionization detector (FID) (Agilent, Santa Clara, CA) as was described elsewhere [29].

2.2.3. HPLC analysis

The phospholipid profile during lipase-catalyzed acidolysis was monitored by high-performance liquid chromatography (HPLC). The HPLC was performed on an Ultimate 3000 DIONEX chromatograph equipped with a DGP-3600A dual-pump fluid control module, a TCC-3200 thermostat column compartment, and a WPS-3000 auto-sampler, photodiode array detector (Olten, Switzerland) and Corona™ Charged Aerosol Detector (CAD) from ESA Biosciences (Chelmsford, MA) with the following parameters: acquisition range—100 pA, digital filter set to none, N_2 pressure—35 psi was used. The system and data acquisition were carried out using the Chromeleon 6.80 software (Dionex Corporation). Analysis was carried out using a Betasil DIOL 5 μm column (Thermo, 150 mm × 2.1 mm). The injection volume was 15 μL in all of the experiments and the cooling temperature for the samples was 20 °C. The column temperature was maintained at 30 °C. The total time analysis was 22 min. The gradient had a constant flow of 1.5 mL/min. Solvent A (1% HCOOH, 0.1% TEA in water), solvent B (hexane) and solvent C (2-propanol) were used in a gradient mode starting with 0/43/57 (%A:%B:%C (v/v/v)), at 5 min = 3/40/57, at 8 min = 10/40/50, at 13 min = 10/40/50, at 13.1 min = 0/43/57 and at 22 min = 0/43/57. The calibration curves for PC (M_w 777 g/mol) and LPC (M_w 508 g/mol) were linear in the range 0–4.14 nmol. Response of the detection method was fitted to the linear model ($y = 31.453x$, $R^2 = 0.998$) for PC as well as for LPC ($y = 24.980x$, $R^2 = 0.999$). The calibration curves for incorporation degree of CLA into PC and LPC were determined on photodiode array detector at 235 nm. At this wavelength conjugated dienes exhibit a high absorption whereas other unsaturated acid residues in PL show no absorption. Response of the detection method was fitted to the linear model for 1,2-di(conjugated) linoleoyl-*sn*-glycero-3-phosphocholine (M_w 782 g/mol, $y = 16.889x$, $R^2 = 0.999$) as well as for 1-(conjugated) linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (M_w 520 g/mol, $y = 2\ 0.496x$, $R^2 = 0.999$).

The incorporation of CLA in the *sn*-1 position of PC and LPC (%) were calculated as follows:

$$PC_{CLA} = \frac{\text{the moles of CLA in PC}}{\text{the moles of LPC}} \times 100\% \quad (1)$$

$$LPC_{CLA} = \frac{\text{the moles of CLA in LPC}}{\text{the moles of LPC}} \times 100\% \quad (2)$$

The effective incorporation of CLA (%) was calculated according to following equation:

$$E_{CLA} = \frac{\text{the moles of CLA in PC} + \text{the moles of CLA in LPC}}{\text{the moles of initial PC}} \times 100\% \quad (3)$$

Effective incorporation of 100% indicates that all native acyl residues in the *sn*-1 position have been exchanged for CLA residues. Results greater than 100% indicate that the additional incorporation occurred into the *sn*-2 position.

The content of PC and LPC in the phospholipid profile was determined based on the HPLC analysis. The content of *sn*-glycero-3-phosphocholine (GPC) (%) was calculated according to the equation:

$$\text{GPC} = \text{the moles of initial PC} - (\text{the moles of PC at a given reaction time} + \text{the moles of LPC at a given reaction time}) \quad (4)$$

2.3. Isolation of phosphatidylcholine from egg yolk

A crude phospholipids from egg yolk were isolated from hen eggs obtained from poultry farm "Ovopol" (Nowa Sól, Poland). The extraction process was carried out on a semi-technical scale using the equipment from Wroclaw Technology Park. Eggs were dried in the drying chamber at inlet air temperature of 185 °C ± 5 °C and at outlet air temperature of 70 °C ± 2 °C. The powder was extracted with ethyl alcohol in a tank equipped with a mechanical stirrer