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Lipase Catalyzed Acidolysis for Efficient Synthesis of Phospholipids Enriched with Isomerically Pure *cis*-9,*trans*-11 and *trans*-10,*cis*-12 Conjugated Linoleic Acid

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Abstract: The production of phospholipid (PL) conjugates with biologically active compounds is nowadays an extensively employed approach. This type of phospholipids conjugates could improve bioavailability of many poorly absorbed active compounds such as isomers of conjugated linoleic acid (CLA), which exhibit versatile biological effects. The studies were carried out to elaborate an efficient enzymatic method for the synthesis of phospholipids with pure (>90%) *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers. For this purpose, three commercially available immobilized lipases were examined in respect to specificity towards CLA isomers in acidolysis of egg-yolk phosphatidylcholine (PC). Different incorporation rates were observed for the individual CLA isomers. Under optimal conditions: PC/CLA molar ratio 1:6; *Rhizomucor miehei* lipase loading 24% wt. based on substrates; heptane; DMF, 5% (v/v); water activity (a_w), 0.11; 45 °C; magnetic stirring, 300 rpm; 48 h., effective incorporation (EINC) of CLA isomers into PC reached ca. 50%. The EINC of CLA isomers was elevated for 25–30% only by adding a water mimic (DMF) and reducing a_w to 0.11 comparing to the reaction system performed at a_w = 0.23. The developed method of phosphatidylcholine acidolysis is the first described in the literature dealing with isometrically pure CLA and allow to obtain very high effective incorporation.

Keywords: conjugated linoleic acid; phosphatidylcholine; structured phospholipids; acidolysis; lipase; lipozyme; effective incorporation; biocatalysis

1. Introduction

Phospholipids (PLs) are important biomolecules for the functioning of living organism, as they are the main component of lipid cytoplasmic membranes ensuring membrane fluidity and mechanical strength. Moreover, some class of PLs acts as mediators in signal transduction through membranes. Thanks to their amphiphilic nature, PLs have the ability to accumulate at the interface between two phases (e.g., oil and water) reducing interfacial tension, facilitate the formation of emulsions and stabilize them. PLs have found broad application in food, cosmetics, and pharmaceutical industries as emulsifiers, dispersants, viscosity regulators, wetting, and anti-spattering agents, thus they are an excellent example of a multifunctional compounds [1,2].

Nowadays, due to biocompatibility and biological function of natural PLs, the production of phospholipid conjugates with drugs [3,4] and other biologically active compounds—such as isoprenoid [5–7], lipoic [8], and phenolic acids [9,10]—is an extensively employed approach. This type of phospholipid conjugate plays a role of lipophilic prodrug where glycerophosphocholine is esterified in *sn*-1 or *sn*-2 position with the biologically active molecules, mainly with those that do not occur

naturally in the phospholipid structure. Such lipophilization of biologically active compounds can to overcome the biological barriers, improves bioavailability and the drug safety profile, facilitating formulation development and drug administration [11].

Soybean and egg-yolk phosphatidylcholine (PC) is the most intensively studied phospholipids for structural modifications [12,13]. Naturally occurring glycerophospholipids usually composed of saturated fatty acids, such as stearic or palmitic acid in the *sn*-1 position, while the *sn*-2 position is occupied by unsaturated acids (i.e., oleic, linoleic, and arachidonic) [14]. Structured phospholipids are the products of such modifications, and the process itself involves rearrangement, addition or replacement of fatty acid residues in the *sn*-1 or *sn*-2 position of glycerol backbone with desired fatty acids, for example medium chain saturated acids, to ensure high oxidative stability [15–17], polyunsaturated fatty acids (eicosapentaenoic and docosahexaenoic) that possess health-promoting properties [18–20] or conjugated fatty acids, such as conjugated linoleic acid (CLA) (Table 1). Amongst the PL modification processes, enzymatic methods are currently becoming the most popular because of many advantages (i.e., regioselectivity, substrate specificity, low temperature, and lack of harsh and harmful chemical reagents). For this purpose, phospholipases (PLA_1 or PLA_2) or 1,3-specifiic lipases of microbiological origin are utilized. The latter ones are of special interest because, in contrast to phospholipases, they do not require cofactors and are commercially available in immobilized form [13].

In general, structural modifications of lipophilic region of PLs include a one- or two-step processes [21]. The one-step process involves phosphatidylcholine and acyl donors and it is known as transesterification. Depending on the acyl donor used, two reactions can be distinguished: acidolysis, which utilizes the free fatty acid; or interesterification, where the exchange occurs between PL and another ester. Transesterification is usually characterized by high excess of acyl donor relative to PC, is easy to perform (one-pot reaction) in a relatively short time span compared to a two-step process. Two-step modification involves hydrolysis of the substrate (phosphatidylcholine), isolation of the intermediate product (lysophosphatidylcholine, LPC), and its subsequent esterification with desired acyl donor. It is usually used for modification of the *sn*-2 position catalyzed by PLA_2 [22–25], as the 1-acyl LPC can be easily isolated from the reaction mixture without any concern about migration side reaction that normally occurs for 2-acyl LPC [26]. The two-step process is more complex, but it can obtain a product of high purity [2].

Conjugated linoleic acid is a term that describes positional and geometrical isomers of C-18 fatty acid with two conjugated double bonds. The most commonly used in scientific studies are *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA. *cis*-9,*trans*-11 CLA (rumenic acid)can be found in fats derived from ruminants, because CLA biosynthesis is closely related to the rumen bacteria, mainly *Butyrivibrio fibrisolvens* [27,28]. *trans*-10,*cis*-12 CLA does not occur naturally and it is obtained mainly in alkali-isomerization of linoleic acid. This isomer is produced together with the former one with equimolar ratio [29].

Table 1. Literature evaluation of the enzyme-catalyzed modification of phospholipid with the mixtures of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid isomers

Substrate/Reaction	Enzyme Load [1]	Substrate Molar Ratio	Parameters	INC (%)/Yield (%)/EINC (%)	Reference
Soy PL/acidolysis	20% Lipozyme TL IM	1/5.5 (PL/CLA [2])	Solvent free/ 60 °C/72 h	*ca.* 30% into PL/nd/nd	[30]
Soy PC/acidolysis	7% Lipozyme RM IM	1/5 (PC/CLA [3])	Hexane/40 °C/ 72 h	16% into PC, 8.1% LPC/80.3% PC/EINC = 19%	[31]
Egg-yolk LPC/esterification	Freeze-dried Lecitase 10L 3.3×10^4 U (PLA_2)/11 mg LPC	*ca.* 1/3 (LPC/CLA [4])	Glycerol with formamide/37 °C/6 h/albumin, $CaCl_2$	-/65% PC	[25]
Soy PC/acidolysis	30% PLA_2 immobilized on Amberlite XAD7	1/3 (PC/CLA [5])	Solvent free/ 45 °C/48 h/water addition	30% into PC/21% PC, 74% LPC/EINC = 6%	[32]

Table 1. *Cont.*

Substrate/Reaction	Enzyme Load [1]	Substrate Molar Ratio	Parameters	INC (%)/Yield (%)/EINC (%)	Reference
Soy PL/transesterification	Lipozyme RM IM	PL/CLAEE	Solvent free/ 45 °C/48 h	31% into PL/nd/nd	[33]
GPC/esterification	10% Novozyme 435	1/50 (GPC/CLA [6])	Solvent free/ 40 °C/48 h	-/70% LPC	[34]
Soy PC/acidolysis	15% Lecitase Ultra (PLA_1) immobilized on Duolite A568	1/4 (PC/CLA [2])	Solvent free / 55 °C/24 h	85.8% calculated on *sn*-1 of PC/12.6% PC/ EINC = 11%	[35]
Soy PC/acidolysis	15% Lecitase Ultra (PLA_1) immobilized on Duolite A568	1/4 (PC/CLA [2])	Solvent free/ 50 °C/24 h	90.1% calculated on *sn*-1 of PC/nd/nd	[36]
Soy PC/acidolysis	30% Lipozyme RM IM	1/6 (PC/CLA [5])	Solvent free/ 48 °C/64 h	24% into PL/nd/nd	[37]
GPC/esterification	300 U/g GPC mutant lipase from marine Streptomyces sp. (MAS1-H108A) immobilized on ECR1030 resin	1:40 (GPC/CLA [7])	Solvent free/ 55 °C/48 h	-/89% LPC, 4% PC	[38]
Egg-yolk PC/acidolysis	24% Lipozyme RM IM	1/8 (PC/CLA [8])	Heptane/45 °C/ 36 h/a_w = 0.32, a_w = 0.11 (after 12 h)	33.8% into PC, 50.1% into LPC/39.5% PC, 25.3% LPC/EINC = 39%	[39]
Egg-yolk PC/acidolysis	24% Lipozyme RM IM	1/8 (PC/CLA [9])	Heptane/45 °C/ 36 h/a_w = 0.32, a_w = 0.11 (after 12 h)	42–44% into PC, 62–65% into LPC/34–36% PC, 16–18% LPC/EINC = 42%	[40]
Soy PC/acidolysis	15% Lecitase Ultra (PLA_1) immobilized on Duolite A658	1/12 (PC/CLA [2])	Solvent free/50 °C /24 h/4 d lyophilized PC	72% into PC/<1% LPC and GPC/EINC > 70%	[41]
Soy PC/acidolysis	15% Lecitase Ultra (PLA_1) immobilized on Lifetech™ ECR8804M	1/12 (PC/CLA [2])	Solvent free/ 50 °C/2 h/4 d lyophilized PC	74% into PC/<1% LPC and GPC/EINC > 70%	[42]

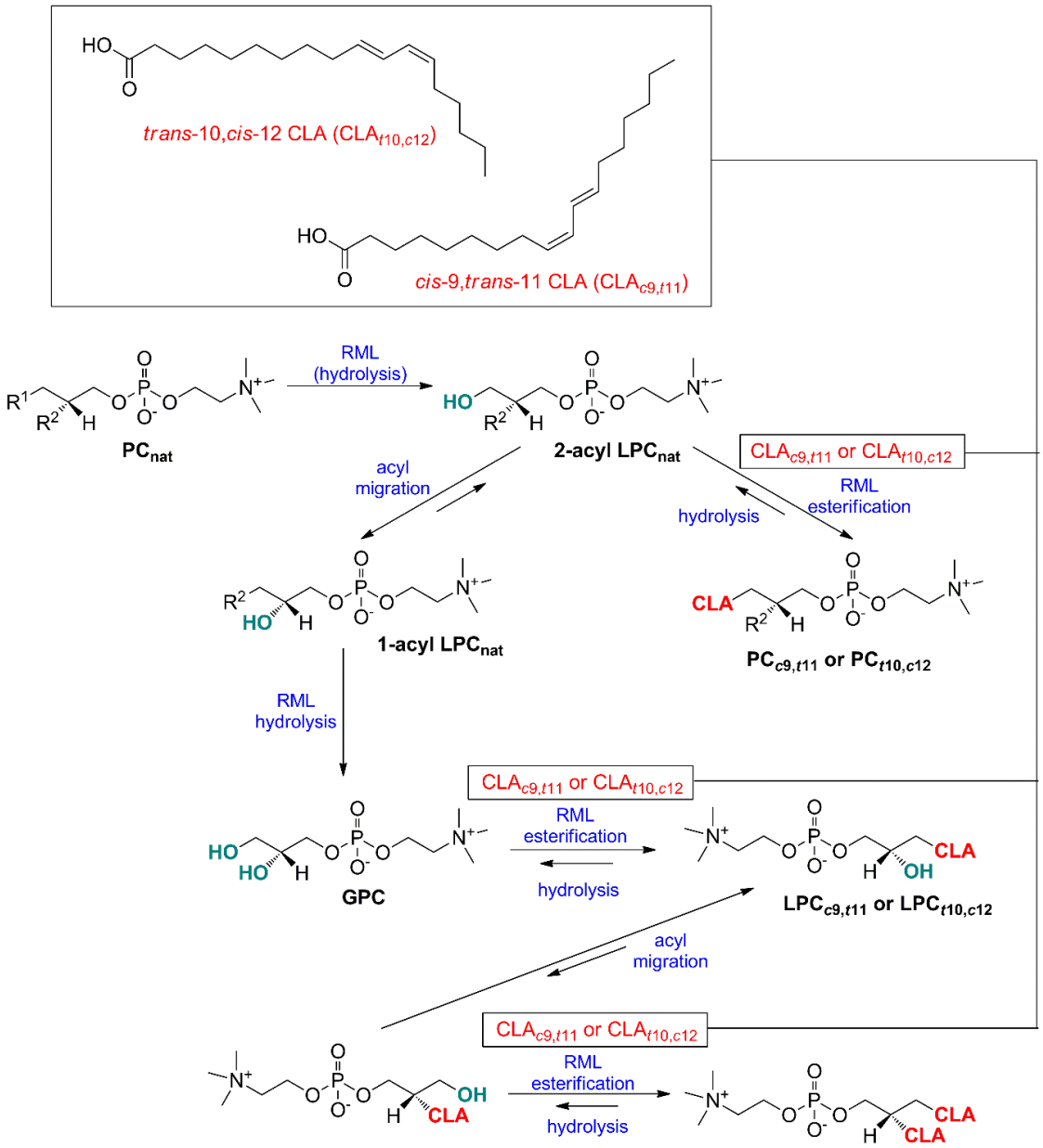
[1] enzyme load with respect to the total weight of substrates; [2] 92% purity; [3] 70.9% purity; [4] 67.1% purity; [5] 80% purity; [6] 77.6% purity; [7] 71% purity; [8] 99.0% purity; [9] CLA concentrates obtained from sunflower oil: 57.1% *t*10,*c*12 CLA, 35.2% *c*9,*t*11 CLA and safflower oil: 58.1% *t*10,*c*12 CLA 33.9% *c*9,*t*11; nd, not determined; CLAEE, conjugated linoleic acid ethyl ester; GPC, *sn*-glycero-3-phosphocholine; INC, incorporation of CLA; EINC, effective incorporation of CLA (Equation (3)).

The literature reports that CLA has diverse effects on physiological function of living organisms including reduction of tumor incidence, altering lipid metabolism, stimulation of immune system, reduction of atherosclerosis risk [28,43]. Recent studies have revealed that conjugates of anticancer drugs, such as pacitaxel [44,45] and gencitabine [46] with CLA, strengthen their activity by increasing stability in blood and facilitating cellular uptake. Also, phosphatidylcholine conjugates of CLA [47,48] as well as lipid nanoparticles based on those PC [49] exhibit antiproliferative activity against malignant skin melanoma, epidermoid carcinoma, leukemia, and breast and colon cancer cells. The aforementioned research had been performed using a mixture of CLA isomers and could not specify the activity of the particular ones. However, the vast majority of the literature data indicate that the biological effects of CLA are isomer specific [50]. For example, *cis*-9,*trans*-11 isomer showed the highest antiproliferation potency in the colon cancer cell line HT-29 [51]. *Trans*-10,*cis*-12 CLA isomer exhibited the widest inhibitory effect, it strongly affected proliferation of colon cancer cells (Caco-2, HT-29, MIP-101) [51,52], rat hepatoma (dRLh-84) [53], breast cancer (MCF-7) [54], and ovarian cancer cell lines (SKOV-3 and A2780) [55].

Since these studies have proven that particular isomers of CLA exhibit different biological activity and PC structured with CLA can be applied as effective anticancer agents, the necessity to develop efficient methods for PC modification with pure CLA isomers is justified. Table 1 summarizes the enzymatic methods for production of structured PL with conjugated linoleic acid. All examples utilized a equimolar mixture of two CLA isomers: *cis*-9,*trans*-11 and *trans*-10,*cis*-12 with the purity in the range

of 71–99%. In this paper research is presented on the *sn*-1,3-specific lipase-catalyzed acidolysis between egg-yolk PC and pure (>90%) *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA. We focused only on the modification of *sn*-1 position, because we wanted to retain most of essential unsaturated fatty acid functionalities which are present in *sn*-2 position of PC molecules. Lipases were chosen as catalysts as they are commercially available in immobilized form, in contrast to PLA_1, which is available only as a solution. This aspect is of great importance for the industries in scaling-up the process. Acidolysisis is a two-step reaction comprising hydrolysis of ester bond in *sn*-1 position ($PC_{nat} + H_2O \rightarrow$ 2-acyl $LPC_{nat} + FA_{nat}$) and further reesterification of previously formed lysophospholipids with desired CLA isomer (2-acyl $LPC_{nat} + CLA \rightleftarrows PC_{CLA} + H_2O$) (Figure 1). The presence of water in the reaction mixture is crucial for the first-step of acidolysis (hydrolysis of PC_{nat}) and also for activation of the immobilized lipase. To get a high initial rate of incorporation of CLA into PC, water activity (a_w) should be at moderately high level [26]. However, under high a_w conditions, side reactions are accelerated. 2-Acyl LPC_{nat} may undergo non-enzymatic migration to 1-acyl LPC_{nat} (2-acyl-$LPC_{nat} \rightleftarrows$ 1-acyl-LPC_{nat}) and subsequent enzymatic hydrolysis to GPC (1-acyl $LPC_{nat} + H_2O \rightleftarrows GPC + FA_{nat}$) which leads to low PLs yield [56,57]. Therefore, a compromise between high incorporation degree and modified phospholipid yield should be taken into consideration during optimization of the lipase-catalyzed acidolysis. Different approaches have been utilized so far to mitigate the hydrolysis and side reaction (acyl migration) and increase yield of modified PLs including: water activity control [39,40] and lowering the water activity during the reaction time [58], prolonged drying of substrate [41,42], applying vacuum for removing excess of water that is formed during reaction [18,34] or addition of hygroscopic proteins, such as albumin or water-mimicking co-solvents [25].

Herein we investigated the influence of water activity and the addition of water-mimicking co-solvent on the incorporation degree of pure *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA and yield of products in order to select appropriate conditions limiting side reactions. The main parameter which was considered herein was "effective incorporation of CLA" (EINC, Equation (3)), which determines the degree of incorporation of CLA into PC and LPC in relation to the initial amount of egg yolk PC used as a substrate for the reaction. This parameter includes both the degree of incorporation of CLA in PC and LPC, as well as the yield of structured phospholipids. Moreover, we assessed the selectivity of lipases with respect to the CLA isomer and molecular species of PCs.



R^1, R^2: fatty acid residues of native phosphatidylcholine
CLA: *cis*-9,*trans*-11 or *trans*-10,*cis*-12 isomer of conjugated linoleic acid

Figure 1. Possible changes occurring during the enzymatic acidolysis of phosphatidylcholine with conjugated linoleic acid isomers.

2. Results and Discussion

2.1. Specificity of Lipases—Choice of Phospholipid Substrate for Acidolysis

In this work, firstly the acidolysis of phosphatidylcholine with CLA isomers was planned to be optimized using synthetic PC. The choice of synthetic substrate with homogeneous palmitic acid residues (PC_{diPA}) was dictated by the utilized method of analysis of modification products. The developed HPLC method was characterized by lower susceptibility to errors caused by the variations in composition of fatty acid residues of the modified PC in case of PC_{diPA} in relation to the natural egg-yolk PC (PC_{nat}).

Commercially available 1,3-regioselective immobilized lipases from *Thermomyces lanuginosus* (TLL), *Rhizomucor miehei* (RML), and non-specific *Candida antarctica* lipase B (CALB) were screened for their specificity in respect to the PC_{diPA}. The mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (CLA_{mix}) was used as an acyl donor and the acidolysis reactions were carried out on rotary shaker under water activity control (a_w = 0.33).

Results presented in Figure 2 show clear differences in reaction course performed with two different phosphatidylcholines. In reaction catalyzed by RML, the highest effective incorporation (EINC) of CLA was achieved for PC_{nat}. In this case, EINC yielded 10.6% after 12 h, whereas PC_{diPA} acidolysis resulted in 7.2% of EINC in 36 h. Other enzymes (CALB and TLL) gave negligible results not exceeding 3%. To take a better look at this phenomenon, the phospholipid distribution in the reaction mixture and the incorporation into particular types of phospholipids (PC_{nat}/PC_{diPA} and LPC_{nat}/LPC_{diPA}) were examined (Table 2). For all catalysts, a higher incorporation degree into PC (INC_{PC}) and into LPC (INC_{LPC}) was observed for PC_{nat} than for PC_{diPA} when used as a substrate. RML was most efficient in producing modified PC_{nat}, determining INC_{PC} and INC_{LPC} at 12.8% and 34.1%, respectively. It has been also observed that in the case of RML and CALB catalyzed acidolysis, the degree of CLA incorporation into LPC produced from PC_{nat} was considerably higher than from PC_{diPA} of ca. 16–20%. What is more, enhanced production of GPC from egg-yolk PC was observed, whereas the amount of LPC predominates among the hydrolysis products derived from PC_{diPA}.

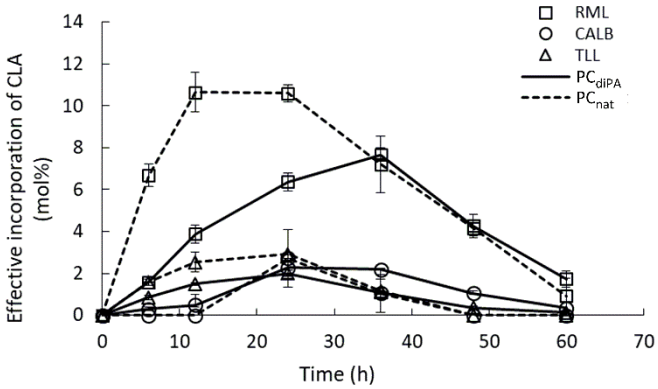


Figure 2. Effect of different phosphatidylcholine substrates on effective incorporation of CLA (EINC) in acidolysis catalyzed by lipases: RML, CALB, TLL. Reaction condition were as follows: PC, 100 mg (0.129 mmol); reaction medium (heptane): 1147 µL; PC/CLA_{mix} molar ratio, 1:6; enzyme dosage, 76 mg; temperature 45 °C. Reactions were performed on rotary shaker (300 RPM). Water activity was controlled at 0.33, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

Table 2. Differences between the effectiveness of the enzyme-catalyzed acidolysis [1] depending on the phosphatidylcholine substrate

Lipase	Incorporation of CLA (mol %)				Phospholipids Distribution (mol %)					
	Substrate PC_{nat}		PC_{diPA}		Substrate PC_{nat}			PC_{diPA}		
	INC_{PC}	INC_{LPC}	INC_{PC}	INC_{LPC}	%PC	%LPC	%GPC	%PC	%LPC	%GPC
RML	12.8 ± 0.9 [2]	34.1 ± 1.1 [6]	5.3 ± 0.5 [4]	8.4 ± 0.3 [9]	35.8 ± 0.6 [11]	17.2 ± 0.8 [16]	47.1 ± 1.3 [19]	57.5 ± 0.2 [13]	31.3 ± 1.1 [18]	11.3 ± 1.2 [21]
CALB	3.8 ± 0.3 [3]	22.7 ± 3.9 [7]	2.6 ± 0.4 [5]	3.4 ± 0.4 [10]	38.2 ± 3.4 [11,12]	11.7 ± 0.6 [20]	50.1 ± 4.0 [19]	53.2 ± 1.0 [14]	30.9 ± 1.2 [18]	15.9 ± 2.2 [22]
TLL	5.9 ± 0.4 [4]	5.3 ± 0.5 [8]	3.1 ± 0.6 [3,5]	nd	37.1 ± 0.4 [12]	13.9 ± 0.3 [17]	49.0 ± 0.7 [19]	42.7 ± 0.5 [15]	30.6 ± 1.1 [18]	26.7 ± 1.5 [23]

[1] Results refers to 24 h of the acidolysis reaction, condition are given in Figure 2. The values are mean ± SD based on three independent experiments. [2–23] Differing letters indicate significant differences between values of particular dependent variable ($p < 0.05$); nd, not detected, <0.01%.

There are several conditions that can lead to such differences in the progress of acidolysis for those two substrates. First, the water activity in both reaction mixtures might be at different level and higher in the case of PC_{nat} acidolysis. This, however, was excluded by water determination. The

measurements revealed that the water content in both substrates as well as in both initial reagent mixtures did not differ from one another.

The second reason might be the lipase specificity. If the catalytic ability of lipase is higher for PC_{nat} than for PC_{diPA}, more products of hydrolysis (LPC and GPC) could appear in the reaction of $PC_{nat.}$ Those products could be further re-esterified with CLA. According to our knowledge, the selectivity of RML to various species of homosubstituted phosphatidylcholines divergent in the type of fatty acids residues has not been investigated so far. However, there are reports in the literature stating that lipases from *R. miehei* promoted hydrolysis of saturated fatty acid (PA and SA) and discriminated against unsaturated residues in TAG from soybean oil [59] and tuna oil [60]. These findings suggest that both phosphatidylcholines (PC_{nat} and PC_{diPA}) should be similarly accepted by *sn*-1 speciffic RML because in this position mainly saturated fatty acids occur. Variations in the course of reactions between PC_{nat} and PC_{diPA} indicate that the type of fatty acid located in the *sn*-2 position may affect the substrate acceptance of lipase. Presumably, those phospholipids in which the *sn*-2 position is occupied predominantly by unsaturated fatty acid (OA and LA) are better accepted.

Another explanation of why less GPC is formed during PC_{diPA} acidolysis might be that there could be no distinctive difference in hydrolysis rate for both PCs, however, the migration of palmitoyl residues in LPC_{PA} is restricted compared to unsaturated acyl residues that greatly predominate in the *sn*-2 position of PC_{nat}. This may be the reason that subsequent hydrolysis (1-palmitoyl LPC + $H_2O \rightleftarrows$ GPC) might be limited during the acidolysis of PC_{diPA}. Research conducted by Sugasini et al. [61] on the acyl migration in four naturally occurring 2-acyl LPCs (PA, OA, EPA, and DHA) stored at various temperatures has proved that, the order of acyl migration rates (from *sn*-2 to *sn*-1) in LPC for both, aqueous buffer and organic solvent ($CHCl_3$:MeOH 2:1 v/v) is 2-PA LPC > 2-OA LPC > 2-EPA LPC > 2-DHA LPC, and higher temperature accelerates migration. It may seem that these findings contradict our conjecture that 2-acyl LPC_{nat} migrates faster than 2-PA LPC, however, as authors have shown, at elevated temperature (>37 °C) differences between migration rates for 2-PA LPC and 2-OA LPC are less significant than for lower temperature. It needs to be pointed out that the behavior of LPCs has not been studied in nonpolar solvents such as heptane or toluene and at 40–60 °C, the conditions the acidolysis reactions are usually carried out. Our observation is an interesting problem and definitely require further investigations. Research on the specificity of lipases relative to the type of fatty acid located in the *sn*-1 and *sn*-2 positions of PC and on the migration of acyl residues in LPC will be continued. Since the effective incorporation of CLA was the highest in reactions using PC_{nat} and RML as catalyst, this combination of reagents and enzyme was chosen for further studies.

2.2. Effect of Mixing Conditions

The problem that is often neglected in optimization of enzymatic process of phosphatidylcholine modification is the mechanical sensitivity of the enzyme support under stirring. The breakdown of the carrier can have a significant effect on the course of the acidolysis reaction, For example if the enzyme efficacy suffers from some diffusion limitations of the substrate, when the catalyst starts to break down, the particle size will diminish and the diffusion problems will decrease. Moreover, it can be a great issue in industrial processes in the separation of phospholipid from reaction mixture, as the fine powders produced from enzyme support can clog the filters of the reactor [62]. To evaluate an influence of stirring condition on effective incorporation of CLA into PL the experiments using magnetic stirrer (300 rpm) or rotary shaker (300 rpm) were carried out, while the other parameters: PC_{nat}/CLA molar ratio of 1:6, an enzyme loading of 24 wt % based on total mass of substrates (PC + CLA) and water activity (0.33) were fixed. Higher EINC of CLA (16.5%) was achieved in 36 h of reaction performed on a magnetic stirrer in contrast to rotary shaker which gives only 10.6% of incorporation after 12–24 h (Figure 3). This may be due to the fact that the magnetic stir bar agitates the enzyme deposits and therefore it intensified diffusion of the reactants between the enzyme and the reaction medium. In the tested shaking intensity, the enzyme bed on the bottom of the vial remained unruffled which apparently limits the mass transfer. It is therefore evident that it is more favorable to

conduct the enzymatic acidolysis with magnetic stirring, hence in these studies, subsequent reactions were carried out on a magnetic stirrer placed in a thermostatically controlled laboratory oven.

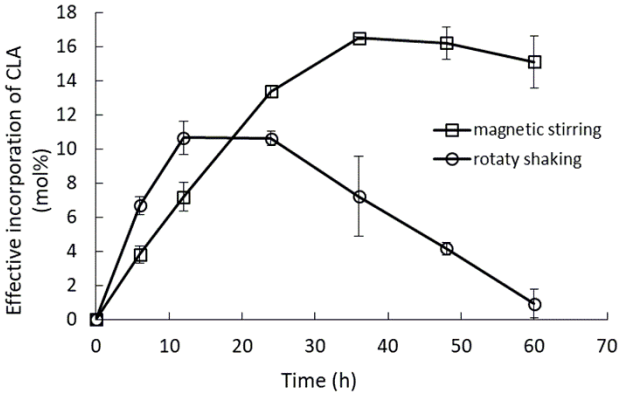


Figure 3. Effect of different stirring conditions on effective incorporation of CLA (EINC) in acidolysis performed on rotary shaker (300 rpm) or magnetic stirrer (300 rpm). Reaction condition were as follows: PC, 100 mg (0.129 mmol); reaction medium (heptane): 1147 μL; PC_{nat}/CLA_{mix} molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C. Water activity was controlled at 0.33, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

Mechanical agitation brings the risk of the carrier break down to overcome this problem methods applying pack bed [63] or rotating bed reactor [64] have been proposed. These however are suitable for large scale processes. In our experiment magnetic stirring seemed to be the most reasonable while no changes in the appearance of the immobilized enzyme were observed in the reaction mixture. In many studies on acidolysis of phosphatidylcholine with conjugated linoleic acid [31] and caprylic acid [30,57,65] catalyzed by immobilized *Rhizomucor miehei* lipase (Lipozyme), magnetic stirring at 300 rpm was applied. None of the authors mentioned the enzyme break down during the reaction.

2.3. Lipases Selectivity to Conjugated Linoleic Acid Isomers

Lipases are widely used as biocatalysts in reactions to modify lipids because of their high regioselectivity in respect to the TAG and PL as well as for their ability to discriminate between individual fatty acids or acyl residues. The substrate specificity of a lipase depends on the carbon chain length, unsaturation degree, positions, and configuration (*cis*/*trans*) of double bonds. The utility of lipases, based on their selectivity, has been proven in numerous studies dealing with esterification transesterification [66] and hydrolysis reactions [67].

To determine the specificity of lipases (RML, CALB, and TLL) relative to the particular CLA isomers, the pure *cis*-9,*trans*-11 CLA (93.8%) and *trans*-10,*cis*-12 CLA (96.2%) isomers were used as an acyl donors in PC_{nat} acidolysis. The experiments were carried out on a magnetic stirrer (300 rpm). All other reaction conditions were unchanged.

The time course of effective incorporation of CLA was similar for individual lipases regardless of applied acyl donor, but it varies between the catalysts (Figure 4). The highest effective incorporation for RML was achieved in 12 h, and it reached 19.9% for *cis*-9,*trans*-11 CLA and 17.2% for *trans*-10,*cis*-12 CLA. The EINC produced by CALB came to the maximum at 24 h reaction giving 10.0% for *cis*-9,*trans*-11 CLA and 9.2% for *trans*-10,*cis*-12 CLA. TLL have shown the greatest differences in reaction course between two isomers. The corresponding EINC of *cis*-9,*trans*-11 CLA was 7.0% after 24 h and EINC for *trans*-10,*cis*-12 CLA was 3.7% after 12 h. This results are in accordance with the literature data, which indicates that TLL exhibits higher selectivity to *cis*-9,*trans*-11 CLA in the esterification of *sn*-glycero-3-phosphocholine [34].

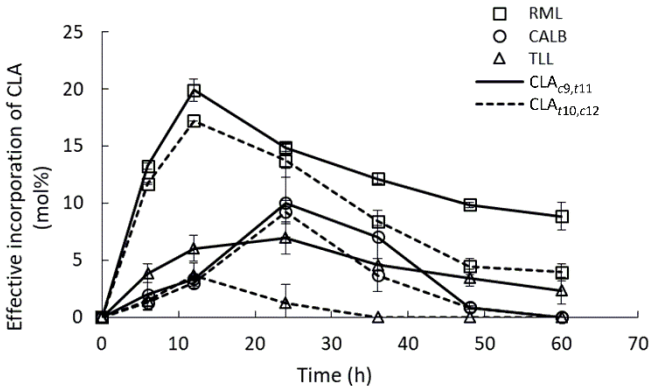


Figure 4. Effect of different conjugated linoleic acid isomers ($CLA_{c9,t11}$ and $CLA_{t10,c12}$) on effective incorporation (EINC) in acidolysis. Reaction condition were as follows: PC_{nat}, 100 mg (0.129 mmol); reaction medium (heptane): 1147 µL; PC_{nat}/CLA molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C; magnetic stirring (300 rpm). Water activity was controlled at 0.33, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

To inspect the acidolysis products, the incorporation of CLA isomers into PC and LPC as well as the phospholipid distribution after 24 h of the reaction were analyzed. Table 3 shows that RML more efficiently incorporated the *trans*-10,*cis*-12 isomer (34.8%) than *cis*-9,*trans*-11 (29.8%) into PC, (differences were statistically significant), while the content of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA in LPC was similar. In the case of CALB, there were no significant differences in the degree of incorporation of individual isomers either to the PC nor to the LPC. It can also be seen that the degree of hydrolysis was markedly higher for RML-catalyzed acidolysis when *trans*-10,*cis*-12 CLA was applied. The corresponding LPC and GPC content in phospholipid fraction reached 9.1% and 67.8% for *trans*-10,*cis*-12 CLA, whereas *cis*-9,*trans*-11 CLA gave a higher ratio of LPC (12.8%) and a lower amount of GPC (60.3%). This demonstrates that greater effective incorporation of the *cis*-9,*trans*-11 isomer in the RML-catalyzed acidolysis was in fact the result of differences in hydrolysis rates between processes employing the *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA as the acyl donor.

Table 3. Differences between the effectiveness of phosphatidylcholine acidolysis [1] depending on the selectivity of lipase to conjugated linoleic acid isomers

Lipase	Incorporation of CLA Isomers (mol%)				Phospholipids Distribution (mol%)					
	Substrate *cis*-9,*trans*-11 CLA		*trans*-10,*cis*-12 CLA		Substrate *cis*-9,*trans*-11 CLA			*trans*-10,*cis*-12 CLA		
	INC_{PC}	INC_{LPC}	INC_{PC}	INC_{LPC}	%PC	%LPC	%GPC	%PC	%LPC	%GPC
RML	29.8 ± 0.1 [2]	52.8 ± 1.5 [7]	34.8 ± 1.0 [5]	55.1 ± 1.3 [7]	27.0 ± 0.3 [11]	12.8 ± 0.1 [15]	60.3 ± 0.1 [21]	23.1 ± 2.8 [13]	9.1 ± 0.1 [18]	67.8 ± 2.9 [24]
CALB	12.5 ± 2.0 [3]	38.7 ± 15.0 [8]	12.4 ± 0.1 [3]	17.3 ± 1.3 [9]	30.6 ± 1.7 [12]	26.2 ± 0.1 [16]	43.2 ± 1.8 [22]	37.7 ± 0.2 [14]	23.4 ± 2.4 [19]	38.9 ± 2.1 [25]
TLL	8.8 ± 0.8 [4]	17.9 ± 0.5 [9]	1.4 ± 0.4 [6]	9.7 ± 1.0 [10]	29.5 ± 1.0 [12]	20.2 ± 0.7 [17]	50.3 ± 0.3 [23]	25.9 ± 4.5 [11,13]	10.6 ± 1.5 [20]	63.5 ± 6.0 [21,24]

[1] Results refers to 24 h of the acidolysis reaction. condition are given in Figure 4. The values are mean ± SD based on three independent experiments. [2–25] Differing letters indicate significant differences between values of particular dependent variable ($p < 0.05$).

Assuming that the initial hydrolysis rate of PC_{nat} ($PC_{nat} + H_2O \rightarrow LPC_{nat}$) is similar for both reactions (with *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA), and RML shows higher specificity to *trans*-10,*cis*-12 CLA under the applied acidolysis conditions, the rate of esterification reaction $LPC_{nat} + CLA_{t10,c12} \rightarrow PC_{t10,c12} + H_2O$ is faster than $LPC_{nat} + CLA_{c9,t11} \rightarrow PC_{c9,t11} + H_2O$. Therefore, in the former reaction, more water is produced—thus the hydrolysis rate of PC and LPC is elevated resulting in the formation of hydrolysis products (LPC and GPC) in a greater extend if the water is not removed efficiently from the reaction mixture through the water activity stabilization system. The obtained results contrast with the literature data. Studies on the esterification reaction of vanillin alcohol [68] and *sn*-glicero-3-phosphocholine [69] with an equimolar mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA showed the weak preference of RML to *cis*-9,*trans*-11 isomer. 8 Similar preference was observed in the

reaction of individual CLA isomers with *n*-butanol [69]. In turn, available literature data indicate higher selectivity of CALB to the *trans*-10,*cis*-12 CLA than to *cis*-9,*trans*-11 CLA in reaction with ethanol [70], *n*-butanol [69], glycerol [71], and *sn*-glycero-3-phosphocholine [34]. In our studies, only the RML showed preference to the *trans*-10,*cis*-12 isomer, as evidenced by a higher degree of incorporation into PC, whereas CALB seemed to be more selective to *cis*-9,*trans*-11 in the esterification of GPC (GPC + $CLA_{c9,t11} \rightarrow LPC_{c9,t11}$) which is confirmed by the higher incorporation degree of *cis*-9,*trans*-11 CLA into LPC (Table 3). The reason for such inconsistencies between our results and literature data may be the fact that our experiments were carried out individually for each isomers, in separate reaction vessels. Therefore, the reaction was non-competitive between *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. Our previous studies on the transesterification of egg-yolk PC with CLA concentrates obtained from safflower and sunflower oil showed no discrepancy in the ratio of CLA isomers between concentrates used as the acyl donors and acyl residues of phospholipid product of acidolysis [40]. This would indicate that in the case of RML, there is no preference for CLA isomers. A similar lack of specificity was observed for this lipase in hydrolysis of CLA glycerides [72]. It is also important to emphasise that in all quoted literature cases, the conditions of the experiments were different and specific for each case. Since the enzyme exhibited different activities and specificities depending on the reaction type, concentration of reagents, medium, temperature, and other parameters, that may have an effect on enzyme properties and complicates the comparison of the results.

2.4. Effect of Water Activity

In order to investigate the effect of the reduction of a_w on the efficiency of PC_{nat} acidolysis with the *cis*-9,*trans*-11 and *trans*-10,*cis*-9 isomers, reactions were carried out at a_w = 0.11, 0.23, and 0.33. Decreasing the water activity affected the course of the acidolysis, and the effective incorporation of CLA declined in the series 0.23 > 0.33 > 0.11 (Figure 5). Our previous studies have shown that the optimal water activity range for effective PC acidolysis with CLA is 0.23–0.43 [39]. In these experiments maximal EINC for both isomers was achieved at a_w = 0.23 and it reached 21.6% and 24.2% in the 48 h of reaction, respectively for *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA. Therefore, lowering a_w from 0.33 to 0.23 allowed us to obtain modified PL more efficiently. Very low content of water (a_w = 0.11) inhibited the hydrolysis reaction, which resulted in the lowest effective incorporation of *cis*-9,*trans*-11 CLA (10.9%) and *trans*-10,*cis*-12 CLA (10.4%). Poor performance of the reaction at 0.11 a_w could also be a consequence of enzyme activity loss. Limited water availability affects activity of enzyme, since there is a specific critical threshold of a_w required to maintain the adequate hydration state of enzyme protein in which active three-dimensional conformation is preserved [73]. However, the minimal water content in the reaction mixture for maintaining enzyme activity is lower for lipases than for phospholipases [74], therefore the effective transesterification is possible even at very low water activity. For example, a_w = 0.064was the optimum for incorporation of heptadecanoic acid into PC via transesterification utilizing *Rhizopus arrhizus* lipase immobilized by adsorption on a polypropylene support [53].

Table 4 presents the effect of water activity on PL distribution and incorporation of CLA into PL after 48 h of the acidolysis performed with *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA. The inhibition of hydrolysis under restricted a_w is confirmed in the phospholipid profile of the reaction mixture. In the reaction carried out with a_w = 0.11 the degree of PC hydrolysis expressed as %GPC was the smallest–29.47% and 11.60% for *cis*-9,*tans*-11 and *trans*-10,*cis*-12 CLA, respectively. In view of obtained results, it can be seen that %GPC increases at elevated water content, while the PC content decreases, as was reported in other papers [32,75,76].

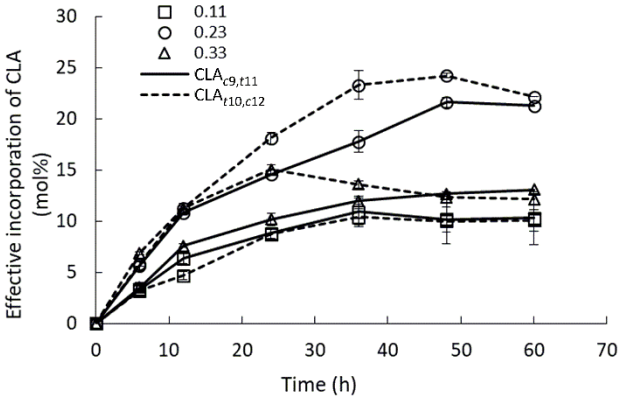


Figure 5. Effect of water activity on effective incorporation (EINC) of CLA$_{c9,t11}$ and CLA$_{t10,c12}$. Reaction condition were as follows: PC, 100 mg (0.129 mmol); reaction medium (heptane): 1147 µL; PC$_{nat}$/CLA molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C; magnetic stirring (300 rpm). Water activity was controlled at 0.11, 0.23, and 0.33, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

Table 4. Differences between the effectiveness of phosphatidylcholine acidolysis [1] depending on the water activity in reaction medium

Lipase	Incorporation of CLA Isomers (mol %)				Phospholipids Distribution (mol %)					
	Substrate *cis*-9,*trans*-11 CLA		*trans*-10,*cis*-12 CLA		Substrate *cis*-9,*trans*-11 CLA			*trans*-10,*cis*-12 CLA		
	INC$_{PC}$	INC$_{LPC}$	INC$_{PC}$	INC$_{LPC}$	%PC	%LPC	%GPC	%PC	%LPC	%GPC
0.11	4.9 ± 0.1 ^{2}	45.5 ± 6.7 ^{7}	4.7 ± 0.4 ^{2}	44.7 ± 2.2 ^{7}	54.3 ± 0.6 ^{10}	16.2 ± 0.2 ^{16}	28.0 ± 1.7 ^{20}	72.6 ± 2.0 ^{13}	15.9 ± 0.5 ^{16}	11.6 ± 2.5 ^{23}
0.23	10.6 ± 0.2 ^{3}	85.9 ± 2.9 ^{8}	24.9 ± 1.2 ^{5}	87.5 ± 1.8 ^{8}	46.6 ± 1.1 ^{11}	20.0 ± 0.2 ^{17}	33.5 ± 1.2 ^{21}	63.3 ± 2.8 ^{14}	22.3 ± 0.9 ^{18}	14.4 ± 3.7 ^{23}
0.33	6.4 ± 0.1 ^{4}	24.4 ± 0.8 ^{9}	11.8 ± 0.3 ^{6}	42.0 ± 0.8 ^{7}	36.1 ± 0.5 ^{12}	15.5 ± 0.8 ^{16}	48.0 ± 0.8 ^{22}	18.3 ± 0.8 ^{15}	8.7 ± 0.3 ^{19}	73.6 ± 3.0 ^{24}

[1] Results refers to 48 h of the acidolysis reaction. condition are given in Figure 5. The values are mean ± SD based on three independent experiments. $^{2-24}$ Differing letters indicate significant differences between values of particular dependent variable ($p < 0.05$).

A significant difference between the distribution of phospholipids in the reactions carried out with particular isomers can be noticed. *trans*-10,*cis*-12 CLA was incorporated in a greater extend into both PC and LPC at 0.23 and 0.33 water activities than *cis*-9,*trans*-11 CLA. The highest degree of INC$_{PC}$ was achieved in water activity of 0.23 and it was 24.95% for *cis*-9,*trans*-11 CLA and only 10.64% for *trans*-10,*cis*-12 CLA. No significant differences in the degree of INC$_{PC}$ between the isomers occurred for a_w = 0.11. CLA was incorporated to LPC in a very high extend and INC$_{LPC}$ reached almost ca. 90% for reaction carried out in a_w = 0.23. No significant differences in INC$_{LPC}$ was observed between the isomers in case of a_w = 0.11 and 0.23. The obtained results are consistent with the literature data, which also proved that the highest degree of incorporation into PL in the RML-catalyzed transesterification were obtained in reduced water activity, which allows limiting the hydrolysis of phospholipids. For example, acidolysis of PC$_{diPA}$ with lipoic acid carried out in toluene at water activity of 0.11 resulted in incorporation yield of 41 mol %, whereas at a_w = 0.43 gave only 10% of the desired product [8].

2.5. Effect of Water-Mimicking Co-Solvent

Studies have shown that the addition of a water-mimicking co-solvents (formamide or DMF) [77,78], may increase the efficiency of lipase catalyzed reactions. A part of the water which is essential for lipase activation can be replaced with other polar solvents characterized by high dielectric constant having the ability to form hydrogen bonds with enzyme protein, but which are not a substrate for hydrolysis [79]. In this section, the effect of reduction of a_w to 0.11 was investigated with the simultaneous addition of DMF to the reaction mixture. The acidolysis was performed with the addition of 5% or 10% v/v of DMF based on the total volume of the reaction mixture and the results were compared with those obtained without the addition of DMF.

The addition of 5% of DMF to the reaction mixture increased the effective incorporation of both CLA isomers by over 30% compared to experiments performer at a_w = 0.11 (Figure 6). Using 5% of DMF at a_w = 0.11 the incorporation of *trans*-10,*cis*-12 CLA and *cis*-9,*trans*-11 CLA reached 52.84% and 46.61% (48 h), respectively. So far, it has been the best result obtained in these studies.

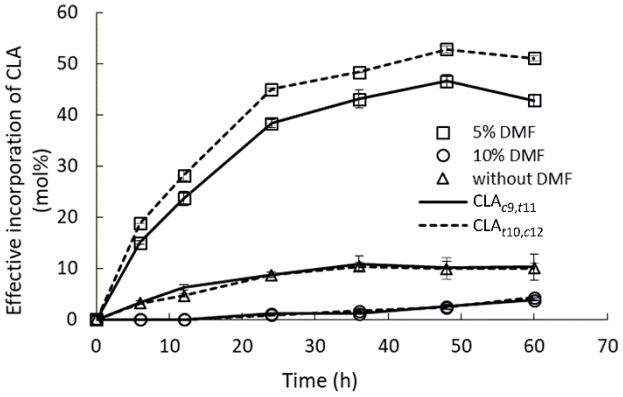


Figure 6. Effect of dimethylformamide on effective incorporation (EINC) of CLA$_{c9,t11}$ and CLA$_{t10,c12}$. Reaction condition were as follows: volume of the reaction mixture, 1.5 mL; reaction medium (heptane); DMF, 5% or 10% v/v; PC, 100 mg (0.129 mmol); PC$_{nat}$/CLA molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C; magnetic stirring (300 rpm). Water activity was controlled at 0.11, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

Positive effect of water mimics (formamide, DMF, N,N-dimethylacetamide, propylene glycol, ethylene glycol) on efficiency of synthesis of structured phospholipids catalysed by lipase and phospholipase was also confirmed by other authors [25,78,80]. Esterification of LPC with CLA mediated by PLA_2 was conducted with addition of small amount of water. Replacement of water with formamide dramatically increased the yield of PC_{CLA} from 2.1% to 45.8% [25]. Furthermore, it has been shown that formamide is an effective polar solvent in synthesis of PC enriched with polyunsaturated fatty acids (PUFA) by PLA_2 and lipase from *Mucor miehei* [80].

The addition of 10% of DMF decreased EINC, which did not exceed even 4% (60 h) for both isomers (Figure 6). Therefore, elevated concentration of DMF diminish the acidolysis effectiveness in relations to PC modification performed at a_w = 0.11 without any co-solvent, in which the highest effective incorporation of CLA (ca. 11%) was achieved at 36 h.

The foregoing observations are in accordance with the aforementioned studies of Yamamoto et al., who observed that doubling the optimal concentration of DMF inhibited PLA_2 activity and thus resulted in a vast decrease in the esterification yield of LPC to only a few percent [25]. It has also been confirmed that lipase activity is reduced by half in the presence of 20% of DMF, while 60% of these solvent caused nearly complete loss of activity [81].

Based on phospholipid distribution in individual reaction mixtures, it can be concluded that the addition of 5% of DMF caused a significant acceleration of hydrolysis compared to the other variants. For example, the GPC content carried out using *cis*-9,*trans*-11 CLA as a substrate, was 48.2% in the final stage of the reaction (60 h), whereas for *trans*-10,*cis*-12 CLA it reached 45.3%. The degree of hydrolysis at the same reaction stage was only 12.2% and 3.7% in acidolysis performed with 10% DMF, for the corresponding isomers (data not shown). The PC content decreased the fastest for 5% DMF and there were no significant differences between reaction conducted with particular isomers at any concentration of co-solvent (Figure 7A and 7B). %LPC reached equilibrium after 6 h and stayed constant at ca. 20%.

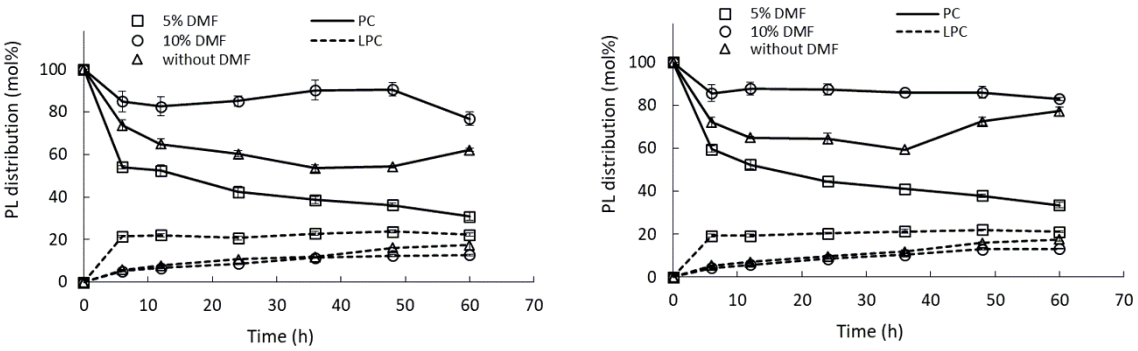


Figure 7. Effect of dimethylformamide on PL distribution (%PC and %LPC) in acidolysis performed with (**a**) $CLA_{c9,t11}$ and (**b**) $CLA_{t10,c12}$. Reaction condition were as follows: volume of the reaction mixture, 1.5 mL; reaction medium (heptane); DMF, 5% or 10% v/v; PC, 100 mg (0.129 mmol); PC_{nat}/CLA molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C; magnetic stirring (300 rpm). Water activity was controlled at 0.11, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

The dependence of the degree of incorporation of individual isomers into PC (Figure 8A) was also investigated in this study. Small concentration of DMF (5%) increased INC_{PC} for ca. 20% for *cis*-9,*trans*-11 CLA and 30% for *trans*-10,*cis*-12 CLA compared to reaction system without co-solvent, whilst 10% DMF decreased the incorporation. INC_{PC} reached a plateau at 24 h and approx. 40% and 30% of incorporation was obtained for *trans*-10,*cis*-12 and *cis*-9,*trans*-11 CLA, respectively. This suggested that RML favored esterification of 2-acyl LPC with the *trans*-10,*cis*-12 isomer in the reaction mixture. However, no specificity was observed using higher DMF concentration as in the case of decreasing water activity (Table 4).

Therefore, it can be concluded that the selectivity of RML to *trans*-10,*cis*-12 isomer of CLA diminishes with the water activity. Water availability or water-mimicking solvents may alter the number of hydrogen bonds formed with the enzyme protein and therefore significantly affect the structural flexibility of enzyme, hence substrate acceptability. In organic solvents (e.g., heptane) and under low water accessibility, lipases usually exhibit higher structural rigidity, thus displaying higher selectivity, as they are more capable of accepting narrow spectrum of substrates. Our observations are not consistent with this principle. However, the literature has presented contradictory examples on whether the enhanced flexibility of the lipase might increase or decrease the selectivity [82].

The time course of the incorporation of CLA isomers into LPC was analyzed as well (Figure 8B). The shape of INC_{LPC} curves varied considerably depending on whether the reactions were carried out with or without addition of co-solvent. INC_{LPC} increased throughout the entire reaction time reaching almost 90% after 60 h (5% DMF). No differences in RML specificity for CLA isomers were observed for incorporation to LPC.

Since RML shows high specificity to the *sn*-1 position of phospholipids [83], LPC_{CLA} in the reaction mixture is formed mainly by esterification of GPC, which is the product of total hydrolysis of PC_{nat} resulting from the migration of acyl residues from the *sn*-2 to *sn*-1 position and further hydrolysis of 1-acyl LPC. Taking into consideration the similar rate of production of GPC, thereby comparable concentration of GPC in reaction performed with both CLA isomers, we conclude that RML shows no selectivity to any of CLA isomers in GPC esterification. So far, no literature data are available in this field.

Due to obtaining the highest effective incorporation of CLA in the experiment using DMF, further studies on the effect of dimethylformamide concentration and the addition of other water-mimicking co-solvent are needed and will be performed in the future.

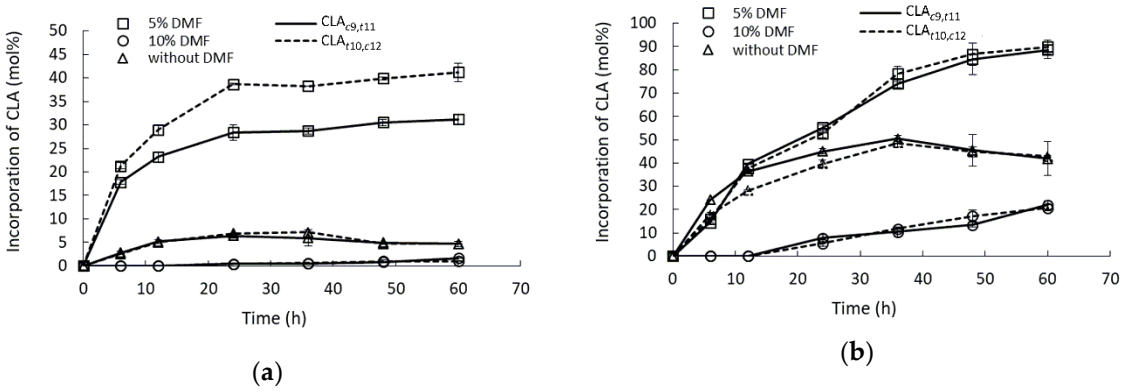


Figure 8. Effect of dimethylformamide on incorporation of CLA into (**a**) PC and (**b**) LPC in acidolysis performed with CLA$_{c9,t11}$ and CLA$_{t10,c12}$. Reaction condition were as follows: volume of the reaction mixture, 1.5 mL; reaction medium (heptane); DMF, 5% or 10% v/v; PC, 100 mg (0.129 mmol); PC$_{nat}$/CLA molar ratio, 1:6; RML dosage, 76 mg; temperature 45 °C; magnetic stirring (300 rpm). Water activity was controlled at 0.11, the reagents were incubated for 24 h before reaction was started. Vertical bars represent SD, n = 3.

2.6. Discussion of Developed Method with the Literature

In many methods of enzymatic preparation of structured phospholipids containing CLA, the objective of the study is to improve the degree of incorporation, whilst phospholipid yield in products mixture is usually neglected. As a consequence the incorporation yield reported by the authors is actually lower when compared to the initial amount of phospholipid substrates, because yield of modified PL always decreases with increasing incorporation of CLA.

Baeza-Jimenez et al. obtained a very high degree of incorporation of CLA into the *sn*-1 position of soy phosphatidylcholine, equal to 90.1%, but did not provide any information on phospholipid efficiency [36]. Similarly, Peng et al. carried out soy PC acidolysis reciving 30% of incorporation, however they did not present any data on efficiency of PL [30]. Hossen and Hernandez reported method of preparation of CLA modified phosphatidylcholine based on acidolysis catalyzed by Lipozyme RM IM [31]. They received structured PL with a high yield (80.3%), but the CLA incorporation was only 16%. However, converting the results into effective incorporation (EINC), disclose that the efficiency of acidolysis catalyzed by Lipozyme TL IM reached 19%. A twice higher INC$_{PC}$ (30%) was obtained by Vikbjerg et al. in PLA$_2$-catalyzed acidolysis in solvent free reaction system, but in this case EINC of CLA was low (ca. 6%) [32].

Recently two publications regarding the acidolysis of soybean PC with conjugated linoleic acid using PLA$_1$ (Lecitase Ultra) immobilized on Lifetech™ ECR8804M, an octadecyl (C18) activated methacrylate resins with a porosity of 350–450 Å [42] and the macroporous resin Duolite A658 [41] have appeared. The authors report that they were able to reduce the hydrolysis of ester bonds in PC to only 1% by using extremely dehydrated lyophilized PC and CLA that was dried over molecular sieves. As a result they obtained a structured PC rich in CLA within 2 h using Lifetech™ ECR8804M immobilized PLA$_1$. This PC product had very high content of CLA yielded 74.4%, which suggest that CLA was incorporated in both *sn*-1 and *sn*-2 positions. It seems that the published procedure is the fastest and most effective approach for PC modification developed so far. However, the method is based on a self-developed catalyst which is not commercially available for industrial application. Moreover, no information was provided on the stability of the lipase immobilized on Lifetech™ during storage as well as in reaction medium. In our research, we used a commercially available immobilized lipase (Lipozyme® RM IM) with known and stable activity. Application of Lipozyme® RM IM in lipid and phospholipid modification has been extensively studied in many publications [13,83].

All methods of PL modifications outlined above utilized a mixture of CLA isomers, which mostly consisted of equal ratio of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers. The literature reported

that individual CLA isomers have a different biological activity and may act synergistically or antagonistically in specific cases [84]. Therefore, to avoid negative interaction between isomers, newly developed phospholipid conjugates should contain isomerically pure CLA molecules. Herein, we presented for the first time the enzymatic strategy for obtaining structured egg-yolk phosphatidylcholine containing pure CLA isomer (>90%). We were able to elevate effective incorporation of the *cis*-9,*trans*-12 and *trans*-10,*cis*-12 CLA isomers to 52.8% and 46.6%, respectively by addition of water-mimicking co-solvent and by controlling water activity during the acidolysis reaction. So far, it is the highest efficiency obtained amongst methods applying commercially available immobilized enzymes.

3. Materials and Methods

3.1. Substrates and Enzymes for Enzymatic Reactions

The phospholipid substrates for enzymatic acidolysis were prepared as follows. Native phosphatidylcholine (PC_{nat}) was isolated from hen egg yolk according to the method described previously [39]. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine was synthesized by the method described elsewhere [47]. The purity of the obtained PCs (>99%) was confirmed via the HPLC method.

The mixture of conjugated linoleic acid (CLA_{mix}) isomers: *cis*-9,*trans*-11, 47.5%; *trans*-10,*cis*-12, 47.7%; all-*cis*,*cis* 2.4%; all-*trans*,*trans* 2.4% was obtained as described in an earlier paper [85]. *cis*-9,*trans*-11 CLA ($CLA_{c9,t11}$) (purity: 93.8%) and *trans*-10,*cis*-12 CLA ($CLA_{t10,c12}$) (purity: 96.2%) were prepared according to the method reported previously [29,85] and analyzed according to the GC method described in Section 3.3.3.

Immobilized lipase from *Rhizomucor miehei* (RML) (Lipozyme® RM IM, >30 U/g) was provided by Fluka (Buchs, Switzerland). Lipase B from *Candida antarctica* immobilized on acrylic resin (CALB) (Novozyme 435, >10,000 U/g) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Immobilized lipase from *Thermomyces lanuginosus* (TLL) (Lipozyme® TL IM, 250 U/g) was supplied by Novozymes A/S (Bagsvaerd, Denmark).

3.2. Chemicals

Standards for chromatography: 1,2-di(conjugated)linoleoyl-*sn*-glycero-3-phosphocholine (PC_{diCLA}) and 1-(conjugated)linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC_{CLA}) were prepared as reported previously [47]; L-α-phosphatidylcholine (≥99%), L-α-lysophosphatidylcholine (≥99%) from egg yolk and fatty acid methyl esters GC standard were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC_{diPA}) and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC_{PA}) were obtained from Avanti® Polar Lipids (Alabaster, AL, USA).

All chemicals and organic solvents were of analytical grade and were supplied by Sigma-Aldrich (St. Louis, MO, USA). The thin layer pre-coated silica gel 60 F_{254}, 0.2 mm plates, silica gel (60 Å, 230–400 mesh) used for column chromatography and all solvents (Merck LiChrosolv® Reag.) for high pressure liquid chromatography were purchased from Merck (Darmstadt, Germany).

3.3. Analytical Methods

3.3.1. Thin-Layer Chromatography (TLC)

Progress of the reaction of enzymatic acidolysis were controlled using the mixture of $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v/v) as a developing system. TLC plates were visualized by immersion in a solution of cerium sulphate (10 g) and phosphomolybdic acid (20 g) in 1 L of 10% (w/w) H_2SO_4 followed by heating.

3.3.2. High Pressure Chromatography (HPLC)

The course of lipase-catalyzed acidolysis was monitored via HPLC. The analysis was performed on an DIONEX UltiMate™ 3000 chromatograph system from Thermo Fisher Scientific™ (Olten, Switzerland) equipped with the photodiode array detector (DAD) and Corona™ charged aerosol detector (CAD) from ESA Biosciences (Chelmsford, MA). Analysis was carried out using a BetaSil™ DIOL column (Thermo Scientific™, 150 × 4,6 mm, 5μm). The injection volume was 15 μL for all analyzed samples. The temperature for the autosampler compartment was 20 °C and column temperature was maintained at 30 °C. The analysis was performed in a gradient mode with 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) The elution program started 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 content of PLs in the reaction mixture and the incorporation degree of CLA into PC and LPC have been assessed on the basis of calibration curves (Supplementary Materials).

The incorporation of CLA in PC and LPC (%) were determined as

$$\mathrm{INC_{PC}} = \frac{\text{the moles of CLA in } sn\text{-1 position of PC}}{\text{the moles of PC}} \times 100\%, \quad (1)$$

$$\mathrm{INC_{LPC}} = \frac{\text{the moles of CLA in } sn\text{-1 position of LPC}}{\text{the moles of LPC}} \times 100\% \quad (2)$$

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

$$\mathrm{EINC} = \frac{\text{the moles of CLA in } sn\text{-1 position of PC} + \text{the moles of CLA in } sn\text{-1 position of LPC}}{\text{the moles of initial PC}} \times 100\%, \quad (3)$$

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 reaction mixture within a specified time duration was determined based on the calibration curves. The content of *sn*-glycero-3-phosphocholine (%GPC) was evaluated according to the equation

$$\%\mathrm{GPC} = \frac{\text{the moles of initial PC} - (\text{the moles of PC} + \text{the moles of LPC})}{\text{the moles of initial PC}} \times 100\%, \quad (4)$$

3.3.3. Gas Chromatography

Obtained *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA were reacted with 1 mL of 4% H_2SO_4 methanol solution at 50 °C for 30 min to obtain conjugated linoleic acid methyl esters (CLAMEs). CLAMEs were then extracted with 1 mL of hexane, washed with saturated NaCl solution and dried with anhydrous $MgSO_4$. CLAME were analyzed via gas chromatography (GC) on an Agilent 6890N apparatus with a flame ionization detector (FID) (Agilent, Santa Clara, CA) fitted with a J&W DB-WAX capillary column (30 m × 320 μm × 0.25 μm film thickness) manufactured by Agilent Technologies (Santa Clara, CA, USA). The temperature program was as follows: 140 °C, 140–250 °C (5 °C/min), final column temperature 250 °C was maintained for 10 min. The split ratio was 25:1. The injector and detector temperature were 250 °C and 280 °C, respectively. Hydrogen was used as the carrier gas with a constant flow 2.5 mL/min. Peaks in GC were identified by comparing their retention times with those of known standards of CLA methyl ester provided by Sigma-Aldrich (St. Louis, MO, USA). The quantitative analysis was carried out based on their peak areas and were calculated using GC ChemStation Version A.10.02.

3.3.4. Water Content

Water content was determined by the Karl Fischer titration method using coulometer model 899 with a generator electrode without diaphragm (Metrohm AG, Switzerland).

3.4. Lipase-Catalyzed Acidolysis

The enzymatic acydolysis of phosphatidylcholine in *sn*-1 position with pure *cis*-9,*trans*-11 and *trans*-10,*cis*-12 conjugated linoleic acid isomers was carried out according to the method described earlier [39]. The following parameters affecting lipase-acidolysis reaction were analyzed during the study: substrate specificity of lipase with respect to the type of phosphatidylcholine and CLA isomers, the mixing conditions (rotary shaking, magnetic stirring), the influence of water activity, and the effect of addition of water-mimicking co-solvent into reaction medium. The reactions were conducted in glass vials at 45 °C, with a mixing intensity of 300 rpm. In each reaction a PC/CLA molar ratio of 1:6 and an enzyme loading of 24 wt % based on total mass of substrates (PC + CLA) were set. The reaction system was composed of 100 mg PC (0.129 mmol), 217 mg of CLA isomer (0.774 mmol), 1147 µL heptane and 76 mg of lipase. Respectively, in the experiments in which the effect of water-mimicking co-solvent–dimethylformamide (DMF) on the efficiency of acidolysis were studied, the reactions were performed in the presence of 5% or 10% (v/v) of DMF based on total volume of the reaction mixture. The acidolysis reaction was carried out in the specific water activity which was maintained by vapor phase equilibration method described by Svensson et al. [76]. Briefly, lipases and substrates were incubated for 48 h in the opened vials placed in sealed containers over heptane in the presence of appropriate saturated salt solution: LiCl (a_w = 0.11), CH_3COOK (a_w = 0.23) or $MgCl_2$ (a_w = 0.33) to maintain desired water activity. After water activity had been set, the containers with reactants were incubated for 1 h at 45 °C for temperature stabilization and the reaction was started by adding enzyme to the mixture containing egg-yolk PC and CLA. Acidolysis was conducted on magnetic stirrer (300 rpm) or rotary shaker (300 rpm) for 60 h. Each experiment was carried out in triplicate. Samples (50 µL) were withdrawn from the reaction mixture at appropriate time intervals: 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, and 60 h; diluted in methanol to a concentration 0.2 mg/mL based on initial concentration of PC in the reaction medium. All samples were stored in −20 °C prior to HPLC analysis. Phospholipid profile of the reaction mixtures and incorporation degree of CLA into PLs were determined by HPLC.

4. Conclusions

In these studies, an improved method for production of structured phospholipids enriched with conjugated linoleic acids isomers was presented. Developed method of phosphatidylcholine acidolysis is the first described in the literature dealing with isomerically pure CLA isomers: *cis*-9,*trans*-11 and *trans*-10,*cis*-12. Lipase from *Rhizomucor miehei* (Lipozyme® RM IM) has been shown as the most potent in acidolysis of phosphatidylcholine in water activity controlled reaction medium and with addition of water-mimicking co-solvent (DMF). Under optimal reaction conditions and water activity control at 0.11 with the addition of DMF (5%) showing water mimic properties it was possible to obtain the effective incorporation of *cis*-9,*trans*-12 and *trans*-10,*cis*-12 CLA of 53% and 47%, respectively. In relation to the acidolysis carried out at a_w = 0.23 the EINC of CLA isomers was elevated for 25–30% only by adding a water-mimicking co-solvent and reducing water activity to 0.11. Moreover, these studies have proved that lipases showed selectivity towards different classes of phosphatidylcholine in respect of fatty acid composition and better accept native egg yolk phosphatidylcholine than synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC_{diPA}). In addition, lipases were indicated to show specificity for CLA isomers in the utilized reaction system.

This study presents the highest effective incorporation of CLA obtained amongst methods applying commercially available immobilized enzymes. The modification product is isomerically pure, which is of great importance due to the fact that CLA isomers show some specificity in biological activity. Modified PC can be used in pharmacy, cosmetics and food industry as a functional component, having

emulsifying and stabilizing properties. The authors envisage a great potential of this structured phosphatidylcholine in development of lipid nanoparticles for use as anticancer drug carriers (e.g., for paclitaxel or gemcitabine), where it has been proven that CLA strengthens their activity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/9/12/1012/s1 Calibration curves for quantification of phospholipid products of the acidolysis reaction.

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Abbreviations

CALB, lipase B from *Candida antarctica*; CLA, conjugated linoleic acid; CLA_{mix}, equimolar mixture of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 of conjugated linoleic acid; $CLA_{c9,t11}$, *cis*-9,*trans*-11 conjugated linoleic acid; $CLA_{t10,c12}$, *trans*-10,*cis*-12 conjugated linoleic acid; INC, incorporation of CLA; DHA, docosahexaenoic acid; DMF, dimethylformamide; EINC, effective incorporation of conjugated linoleic acid; EPA, eicosapentaenoic acid; GPC, *sn*-glycero-3-phosphocholine; INC_{PC}, incorporation of conjugated linoleic acid into phosphatidylcholine; INC_{LPC}, incorporation of conjugated linoleic acid into lysophosphatidylcholine; LA, linoleic acid; LPC; lysophosphatidylcholine; LPC_{CLA}, 1-(conjugated)linoleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine LPC_{nat}, native lysophosphatidylcholine from egg-yolk; $LPC_{c9,t11}$, lysophosphatidylcholine conjugates of *cis*-9,*trans*-11 conjugated linoleic acid; LPC_{PA}, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; OA, oleic acid; PA, palmitic acid; PC, phosphatidylcholine; $PC_{c9,t11}$, phosphatidylcholine conjugates of *cis*-9,*trans*-11 conjugated linoleic acid; PC_{diPA}, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PC_{nat}, native phosphatidylcholine from egg-yolk; $PC_{t10,c12}$, phosphatidylcholine conjugates of *trans*-10,*cis*-12 conjugated linoleic acid; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PL, phospholipid; RML, lipase from *Rhizomucor miehei*; TLL, lipase from *Thermomyces lanuginosus*.

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