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3 Production of phosphatidylcholine containing conjugated linoleic
4 acid mediated by phospholipase A₂
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1 **Abstract**

2 Esterification of lysophosphatidylcholine (LPC) with conjugated linoleic acid
3 (CLA) was carried out using porcine pancreatic phospholipase A₂ (PLA₂). PLA₂ only
4 slightly synthesized phosphatidylcholine containing CLA (CLA-PC) at 2.6% by the
5 addition of water. Addition of formamide in place of water markedly increased the
6 yield of CLA-PC. In addition, synthesis of CLA-PC by PLA₂ was affected by the
7 amount of substrate CLA and PLA₂ in the reaction system. Under optimal reaction
8 conditions using 11 mg LPC, 18 mg CLA, 550 mg glycerol, 50 µL formamide,
9 3.3×10⁴ U PLA₂, and 0.3 µmol CaCl₂ at 37°C for 6 h, the reaction yield of CLA-PC
10 reached 65 mol%. Furthermore, addition of protein such as albumin and casein
11 suppressed the decrease of CLA-PC yield after 6 h. In addition, PLA₂ exhibited the
12 highest activity for the 10*t*,12*c*-CLA isomer among four CLA isomers (9*c*,11*t*-CLA,
13 9*c*,11*c*-CLA, 9*t*,11*t*-CLA and 10*t*,12*c*-CLA), whereas that for 9*c*,11*c*-CLA was the
14 lowest. These results showed that the present esterification system for LPC and CLA
15 by PLA₂ is effective for producing CLA-PC.

16

17 *Keywords:* Phosphatidylcholine, Conjugated linoleic acid, Phospholipase A₂,
18 Esterification, Water mimic

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1 **1. Introduction**

2

3 Conjugated linoleic acids (CLAs) are isomers of linoleic acid (9cis, 12cis-C_{18:2})
4 differing in the position and *cis/trans*-configuration of their conjugated double bond.
5 Recently, CLAs have received attention because of their anti-obesity effect [1],
6 anti-hypertensive effect [2], and anti-cancer effect [3]. In particular, it has been
7 reported that dietary CLA decreases body fat and body mass [4-6].

8 Natural dietary sources of CLA include meat and milk products of ruminants.
9 However, the amount of CLA in these products is very low, at less than 1% of the
10 fatty acid composition of their lipids, and the predominant isomer is 9_c,11_t-CLA.
11 Therefore, CLA is commercially prepared by alkaline isomerization of linoleic acid,
12 resulting in a mixture of several isomers including the two major ones, 9_c,11_t-CLA
13 and 10_t,12_c-CLA. In addition, microbial production of 9_t,11_t-CLA isomer by lactic
14 acid bacteria has also been studied [7, 8]. CLA obtained by these methods is mainly
15 free fatty acid, which shows toxicity at high levels in foods. It is therefore important
16 to prepare triacylglycerol or phospholipids containing CLA for utilization in
17 nutraceutical fields. In particular, phospholipids are useful in many applications, such
18 as food emulsifiers and cosmetics, because of their interfacial activity. In addition,
19 phospholipids containing CLA can be applied to liposome technology used in drug
20 carrier development. Thus, phospholipids containing CLA are an effective lipid form

1 in developing medical applications of CLA.

2 There are many reports describing the preparation of triacylglycerol [9],
3 1,3-diacylglycerol [10] and monoacylglycerol [11] using lipase. However, there are
4 only a few reports describing the preparation of phospholipids containing CLA
5 (CLA-PL) through transesterification using lipase [12]. In contrast, we have already
6 reported on the preparation of phospholipids containing eicosapentaenoic acid at the
7 *sn*-2 position by phospholipase A₂ (PLA₂) [13]. This reaction system with PLA₂ is
8 fundamental in the preparation of CLA-PC.

9 In this study, we investigated the optimum conditions for the esterification of
10 LPC with CLA using PLA₂. Formamide and albumin were effective at regulating the
11 water content in the reaction systems including PLA₂ and resulted in high CLA-PC
12 reaction yield. Furthermore, we demonstrated that PLA₂ exhibits specificity for CLA
13 isomers during esterification.

14

15 **2. Materials and methods**

16

17 *2.1. Materials*

18

19 L- α -Lysophosphatidylcholine from egg yolk was purchased from Wako Pure
20 Chemical Industries, Ltd. (Osaka, Japan). Industrial Phospholipase A₂ (Lecitase 10L)

1 from porcine pancreas was purchased from Novozymes A/S (Bagsvaerd, Denmark).
2 PLA₂ was used after dialysis of Lecitase 10L with cellulose tube in distilled water
3 and freeze-drying. Substrate CLA-mixture (9*c*,11*t*-CLA 33%, 10*t*,12*c*-CLA 34%) was
4 kindly provided by Nisshin OilliO Group, Ltd. (Tokyo, Japan). 9*c*,11*c*-CLA,
5 9*c*,11*t*-CLA, 9*t*,11*t*-CLA and 10*t*,12*c*-CLA isomers were purchased from Cayman
6 Chemical Co. (Ann Arbor, MI, USA).
7 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (DHPC) used as an internal
8 standard was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Bovine
9 serum albumin was purchased from MERCK (Darmstadt, Germany). γ -Globulin from
10 bovine blood was purchased from Sigma Chemical Co. (St. Louis, MO, USA).
11 Casein from milk and gliadin were purchased from Nacalai Tesque, Inc. (Kyoto,
12 Japan) and MP Biomedicals, LLC (Eschwege, Germany), respectively. All solvents
13 and other chemicals used were at least of analytical grade.

14

15 2.2. Esterification

16

17 Typical reaction mixture for synthesis of CLA-PC was LPC 11 mg, CLA 18 mg,
18 PLA₂ 3.3×10⁴ U, glycerol 550 mg, water or formamide 50 μ L and CaCl₂ 0.3 μ mol.
19 Synthetic reaction of CLA-PC was carried out at 37°C, 50-200 rpm in the dark. The
20 reaction was stopped by addition of methanol. Then, chloroform and water were

added to the reaction mixture to adjust chloroform-methanol-water (10:5:3, v/v/v).
The lipid fraction including synthesized CLA-PC, substrate CLA and LPC was
obtained from chloroform layer.

2.3. HPLC analysis of CLA-PC

Synthesized CLA-PC was detected by HPLC system (L-7100, HITACHI, Tokyo, Japan) equipped with a silica gel column (Mightysil Si 60, Kanto Chemical CO., Inc.) (Tokyo, Japan). Lipid fraction separated from reaction mixture as described in 2.2. *Esterification* was injected into HPLC. The mobile phase was consisted of acetonitrile-methanol-sulfuric acid (100:3:0.05, v/v/v) and flow rate was 1.0 mL/min. CLA-PC was detected at 233 nm with diode array detector (HITACH, L-7455, Tokyo, Japan). In this system, retention time of CLA-PC was at around 16 min.

2.4. Gas chromatography analysis of fatty acid composition of CLA-PC

Synthesized CLA-PC was separated by silica gel thin layer chromatography (TLC) using chloroform-methanol-water (65:25:4, v/v/v) as the developmental solvent. Then, fatty acid composition of CLA-PC was analyzed with gas chromatography (GC) after methylation by sodium methoxide methanol solution. GC

1 system was consisted of gas chromatograph (Shimadzu, GC-14B, Kyoto, Japan.)
2 equipped with flame ionization detector and fused silica capillary column,
3 Omegawax 320 (30 m × 0.32 mm i.d.) (Supelco Inc., Bellefonte, PA, USA). Injector,
4 column and detector temperature were 250°C, 200°C and 260°C, respectively. Helium
5 gas was used as carrier gas and its pressure was adjusted to 50 kPa.

6

7 *2.5. Calculation of CLA-PC yield*

8

9 The yield of CLA-PC synthesized from LPC and CLA-mixture was measured by
10 HPLC. The amount of CLA-PC was calculated by the calibration curve using
11 synthesized CLA-PC (MW 273.5). The yield was estimated according to following
12 equation.

13

$$14 \text{ Yield (mol\%)} = \text{CLA-PC (mol)} / \text{LPC (mol)} \times 100$$

15

16 For studies using purified 9_c,11_t-CLA, 10_t,12_c-CLA, 9_t,11_t-CLA and
17 9_c,11_c-CLA isomers, the yield of synthesized CLA-PC was estimated by GC analysis
18 using DHPC as internal standard. That is, an adequate amount of DHPC solution (0.1
19 mg/mL chloroform) was added to lipid fraction separated from reaction mixture. The
20 lipid fraction was applied to silica gel TLC plate with fluorescence dye (Silica gel 60

F₂₅₄, Merck, Darmstadt, Germany) and developed by chloroform-methanol-water (65:25:4, v/v/v). After detection by UV at 254 nm, PC fraction was scraped and was then eluted by chloroform-methanol (3:7, v/v). PC obtained was methylated using sodium methoxide methanol solution and fatty acid composition was analyzed with GC system as described in 2.4. *Gas chromatography analysis of fatty acid composition of CLA-PC*. The yield of synthesized CLA-PC was calculated from the amount of total fatty acid vs. heptadecanoic acid from DHPC.

3. Results and discussion

3.1. Synthesis of CLA-PC

We attempted the synthesis of CLA-PC through esterification of LPC with CLA by PLA₂. As shown in Fig. 1, a new peak with the same retention time (16 min.) as standard PC was observed at 233 nm by HPLC analysis. The peak of LPC was detected at 210 nm on 40 min. To confirm CLA-PC synthesis, the fatty acid composition of the synthesized PC was analyzed by GC (Table 1). 9*c*,11*t*-CLA and 10*t*,12*c*-CLA were detected at 13.9% and 15.1% in the fatty acid composition of the synthesized PC, respectively. These data indicate the synthesis of CLA-PC by PLA₂.

1 3.2. *Effect of formamide on CLA-PC synthesis by PLA₂*

2

3 Esterification of LPC with CLA mediated by PLA₂ was conducted under low
4 water conditions (Fig. 2). However, the yield of CLA-PC was only 2.1 mol% using
5 the reaction conditions of 11 mg LPC, 18 mg CLA, 3.3×10^4 U PLA₂, 550 mg glycerol,
6 0.3 μ mol CaCl₂ and 50 μ L water at 37°C for 48 h. In contrast, addition of formamide
7 in place of water dramatically increased the yield of CLA-PC to 45.8 mol%.

8 Formamide is known as a “water mimic” that activates enzymes instead of water
9 because of its high dielectric constant and ability to form multiple hydrogen bonds
10 with protein [14, 15]. In our previous reports, we showed that formamide is an
11 effective polar solvent in synthesizing PC containing polyunsaturated fatty acids
12 (PUFA) by PLA₂ [13]. That is, formamide promoted the esterification of LPC with
13 PUFA by PLA₂ and suppressed the hydrolysis of PUFA-PC. In the synthesis of
14 CLA-PC, it was suggested that formamide activated PLA₂ and considerably
15 improved the yield.

16

17 3.3. *Optimal amount of formamide*

18

19 We examined the optimal amount of formamide for CLA-PC synthesis. The yield
20 of CLA-PC increased with the amount of formamide and reached a maximum of 48.2

1 mol% at 200 μ L of formamide. However, formamide addition over 200 μ L decreased
2 the CLA-PC yield (Fig. 3). These data indicate that the optimal amount of formamide
3 is 200 μ L for the reaction mixture of 11 mg LPC, 18 mg CLA, 3.3×10^4 U PLA₂, 550
4 mg glycerol, and 0.3 μ mol CaCl₂. In the reaction system without formamide, the
5 CLA-PC yield was 22 mol% (data not shown). This may be because glycerol, which
6 was used to disperse the substrate in the reaction system, also plays a role as water
7 mimic, as previously reported [14].

8

9 *3.4. Optimal amounts of PLA₂, CLA and glycerol*

10

11 To estimate the optimal amount of PLA₂, the synthesis reaction was conducted in
12 the range of 0.275×10^4 U- 1.32×10^4 U (Fig. 4). The yield of CLA-PC increased as the
13 amount of PLA₂ increased and reached a plateau at 0.55×10^4 U. We then examined
14 the effect of the amount of substrate CLA on CLA-PC synthesis by PLA₂. As with
15 PLA₂, the yield of CLA-PC increased dose-dependently up to 72 mg CLA in the
16 reaction system (Fig. 5).

17 Furthermore, optimal amount of glycerol for CLA-PC synthesis was 550 mg (data
18 not shown).

19

20 *3.5. Effect of protein addition*

1
2 Regulation of the water content around PLA₂ is crucial for CLA-PC synthesis in
3 non-aqueous media because water is also produced by the proceeding esterification
4 even if no water is added to the reaction system. In previous studies, regulation of
5 water content in reaction system by water activity control and decompression has
6 been attempted in PC synthesis by PLA₂ [16,17]. On the other hand, some proteins
7 are known to have hygroscopicity [18]. We therefore investigated the effect of protein
8 on synthesis of CLA-PC (Fig. 6.). The addition of albumin, γ -globulin, casein and
9 gliagin increased the CLA-PC yield compared to that without proteins, except for
10 PLA₂ (Fig. 6A). Furthermore, we examined the optimal amount of albumin for
11 CLA-PC synthesis. As shown in Fig. 6B, the addition of albumin in the range of 0.5
12 mg - 10 mg was effective at increasing the yield of CLA-PC after 48 h. In particular,
13 the addition of 1 mg of albumin increased the yield of CLA-PC to 59 mol%. However,
14 the CLA-PC yield decreased by the addition of 100 mg of albumin. This may be
15 because of the high viscosity and a decrease in the water content around PLA₂ due to
16 water absorption by the albumin.

17 We measured the time course of CLA-PC synthesis by PLA₂ (Fig. 7). The yield of
18 CLA-PC rapidly increased, reaching 65 mol% after 6 h. However, synthesis of
19 CLA-PC gradually decreased after 6 h in the reaction system without albumin. In
20 contrast, the CLA-PC yield in the reaction system with albumin was maintained at

1 approximately 60 mol% even after 6 h. This suggested that water absorbed from
2 atmosphere might shift the reaction equilibrium to hydrolysis in the later stage of the
3 reaction, resulting in a decrease of CLA-PC. Albumin may be able to control the
4 water activity in the reaction system. This study indicated, for the first time, that
5 addition of proteins is effective to maintain CLA-PC yield at a high level.

6 7 *3.6. Synthesis of CLA-PC using CLA isomers*

8
9 CLA has several isomers such as *9_c,11_t*-CLA, *10_t,12_c*-CLA, *9_t,11_t*-CLA and
10 *9_c,11_c*-CLA. In addition, physiological activities of CLA, such as the anti-obesity
11 effect [1], anti-hypertensive effect [19], and anti-cancer effect [20], are known to be
12 different among isomers. We therefore examined the substrate specificity of PLA₂ for
13 esterification using commercially available CLA isomers, *9_c,11_t*-CLA, *10_t,12_c*-CLA,
14 *9_t,11_t*-CLA and *9_c,11_c*-CLA (Fig. 8).

15 The reaction yield was the highest for *10_t,12_c*-CLA and reached 55.9 mol% after
16 48 h reaction. Both *9_c,11_t*-CLA and *9_t,11_t*-CLA, PLA₂ exhibited the same specificity.
17 On the other hand, of the four CLA isomers used in this study, *9_c,11_c*-CLA was found
18 to be unsuitable for CLA-PC synthesis by PLA₂. These results indicate that porcine
19 pancreatic PLA₂ has different substrate specificity for CLA isomers in the
20 esterification of LPC.

4. Conclusion

This study clarified that esterification of LPC with CLA by PLA₂ is effective reaction system to produce CLA-PC. Addition of formamide as a water mimic increased the reaction yield of CLA-PC. Furthermore, some proteins were effective additives to suppress hydrolysis of synthesized CLA-PC during reaction. In addition, PLA₂ was indicated to show the specificity for CLA isomers in the present reaction system. CLA-PC is expected as multi-functional lipid with both functions of CLA and PL and to be applied in nutraceutical fields. These results would lead to enzymatic process to produce CLA-PC.

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Figure legends

Fig. 1. HPLC chromatogram. (A) PC standard (dioleoylphosphatidylcholine); (B) reaction product.

Fig. 2. Effect of formamide on CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture (9*c*,11*t*-CLA 33.1%, 10*t*,12*c*-CLA 34.0%), 3.3×10⁴ U PLA₂, 550 mg glycerol, 0.3 μmol CaCl₂, and 50 μL water or 50 μL formamide. The reaction was conducted at 37°C for 48 h.

Fig. 3. Optimal amount of formamide for CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 3.3×10⁴ U PLA₂, 550 mg glycerol, 0.3 μmol CaCl₂, and 25-800 μL formamide. The reaction was conducted at 37°C for 48 h.

Fig. 4. Effect of the amount of PLA₂ on CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 50 μl formamide, 0.3 μmol CaCl₂, and 0.275-5.5×10⁴ U PLA₂. The reaction was conducted at 37°C for 48 h.

1 Fig. 5. Effect of the amount of CLA on CLA-PC synthesis by PLA₂. Reaction
2 mixture: 11 mg LPC, 18-216 mg CLA-mixture, 3.3×10⁴ U PLA₂, 550 mg glycerol, 50
3 μL formamide, 0.3 μmol CaCl₂. The reaction was conducted at 37°C for 48 h.

4

5 Fig. 6. Effect of protein addition on CLA-PC synthesis by PLA₂. (A) Reaction
6 mixture: 11 mg LPC, 18 mg CLA mixture, 550 mg glycerol, 3.3×10⁴ U PLA₂, 50 μL
7 formamide, 0.3 μmol CaCl₂, and 1 mg protein. The reaction was conducted at 37°C
8 for 48 h. (B) Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol,
9 3.3×10⁴ U PLA₂, 50 μL formamide, 0.3 μmol CaCl₂, and 0-100 mg albumin. The
10 reaction was conducted at 37°C for 48 h.

11

12 Fig. 7. Time course of CLA-PC synthesis by PLA₂ in the presence of albumin.
13 Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3×10⁴ U
14 PLA₂, 50 μL formamide, 0.3 μmol CaCl₂, and 1 mg albumin. The reaction was
15 conducted at 37°C for 48 h.

16

17 Fig. 8. Substrate specificity of PLA₂ for CLA isomers in the esterification of LPC.
18 Reaction mixture: 11 mg LPC, 18 mg CLA isomer, 3.3×10⁴ U PLA₂, 550 mg glycerol,
19 50 μL formamide, and 0.3 μmol CaCl₂. The reaction was conducted at 37°C for 48 h.

20

Table 1. Fatty acid composition of substrate LPC, CLA-mixture and synthesized CLA-PC.

	LPC	CLA-mixture	CLA-PC
		(w%)	
C16:0	70.6	6.6	35.6
C16:1	1.9	-	1.1
C18:0	21.3	2.2	16.2
C18:1	5.5	16.8	12.6
C18:2	0.7	4.5	0.7
9 <i>c</i> ,11 <i>t</i> -CLA	-	33.1	13.9
10 <i>t</i> ,12 <i>c</i> -CLA	-	34.0	15.1

Fig.1. HPLC chromatogram. (A) PC standard (dioleoylphosphatidylcholine); (B) reaction product

Fig. 2. Effect of formamide on CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture (9*c*,11*t*-CLA 33.1%, 10*t*,12*c*-CLA 34.0%), 3.3×10⁴ U PLA₂, 550 mg glycerol, 0.3 μmol CaCl₂, and 50 μl water or 50 μl formamide. The reaction was conducted at 37°C for 48 h.

Fig. 3. Optimal amount of formamide for CLA-PC synthesis by PLA_2 . Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 3.3×10^4 U PLA_2 , 550 mg glycerol, $0.3 \mu\text{mol}$ CaCl_2 , and 25-800 μl formamide. The reaction was conducted at 37°C for 48 h.

Fig. 4. Effect of the amount of PLA₂ on CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 50 μ l formamide, 0.3 μ mol CaCl₂, and 0.275-5.5x10⁴ U PLA₂. The reaction was conducted at 37°C for 48 h.

Fig. 5. Effect of the amount of CLA on CLA-PC synthesis by PLA₂. Reaction mixture: 11 mg LPC, 18-216 mg CLA-mixture, 3.3×10^4 U PLA₂, 550 mg glycerol, 50 μ l formamide, 0.3 μ mol CaCl₂. The reaction was conducted at 37°C for 48 h.

Fig. 6. Effect of protein addition on CLA-PC synthesis by PLA₂. (A) Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3×10^4 U PLA₂, 50 μ l formamide, 0.3 μ mol CaCl₂, and 1 mg protein. The reaction was conducted at 37°C for 48 h. (B) Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3×10^4 U PLA₂, 50 μ l formamide, 0.3 mmol CaCl₂, and 0-100 mg albumin. The reaction was conducted at 37°C for 48 h.

Fig. 7. Time course of CLA-PC synthesis by PLA_2 in the presence of albumin. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3×10^4 U PLA_2 , 50 μl formamide, 0.3 μmol CaCl_2 , and 1 mg albumin. The reaction was conducted at 37°C for 48 h.

Fig. 8. Substrate specificity of PLA₂ for CLA isomers in the esterification of LPC. Reaction mixture: 11 mg LPC, 18 mg CLA isomer, 3.3x10⁴ U PLA₂, 550 mg glycerol, 50 µl formamide, and 0.3 µmol CaCl₂. The reaction was conducted at 37°C for 48 h.