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Production of phosphatidylcholine containing conjugated linoleic acid mediated by phospholipase A_2

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Abstract

Esterification of lysophosphatidylcholine (LPC) with conjugated linoleic acid (CLA) was carried out using porcine pancreatic phospholipase A_2 (PLA_2). PLA_2 only slightly synthesized phosphatidylcholine containing CLA (CLA-PC) at 2.6% by the addition of water. Addition of formamide in place of water markedly increased the yield of CLA-PC. In addition, synthesis of CLA-PC by PLA_2 was affected by the amount of substrate CLA and PLA_2 in the reaction system. Under optimal reaction conditions using 11 mg LPC, 18 mg CLA, 550 mg glycerol, 50 μL formamide, 3.3×10^4 U PLA_2, and 0.3 μmol $CaCl_2$ at 37 °C for 6 h, the reaction yield of CLA-PC reached 65 mol%. Furthermore, addition of protein such as albumin and casein suppressed the decrease of CLA-PC yield after 6 h. PLA_2 exhibited the highest activity for the 10*t*,12*c*-CLA isomer among four CLA isomers (9*c*,11*t*-CLA, 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 lowest. These results showed that the present esterification system for LPC and CLA by PLA_2 is effective for producing CLA-PC.

Keywords: Phosphatidylcholine; Conjugated linoleic acid; Phosphalipase A_2; Esterification; Water mimic

1. Introduction

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

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

There are many reports describing the preparation of triacylglycerol [9], 1,3-diacylglycerol [10] and monoacylglycerol [11] using lipase. However, there are only a few reports describing the preparation of phospholipids containing CLA (CLA-PL) through transesterification using lipase [12]. In contrast, we have already reported on the preparation of phospholipids containing eicosapentaenoic acid at the *sn*-2 position by phospholipase A_2 (PLA_2) [13]. This reaction system with PLA_2 is fundamental in the preparation of CLA-PC.

In this study, we investigated the optimum conditions for the esterification of LPC with CLA using PLA_2. Formamide and albumin were effective at regulating the water content in the reaction systems including PLA_2 and resulted in high CLA-PC reaction yield. Furthermore, we demonstrated that PLA_2 exhibits specificity for CLA isomers during esterification.

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2. Materials and methods

2.1. Materials

L-α-Lysophosphatidylcholine from egg yolk was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Industrial PLA_2 (Lecitase 10 L) from porcine pancreas was purchased from Novozymes A/S (Bagsvaerd, Denmark). PLA_2 was used after dialysis of Lecitase 10 L with cellulose tube in distilled water and freeze-drying. Substrate CLA-mixture (9*c*,11*t*-CLA 33%, 10*t*,12*c*-CLA 34%) was kindly provided by Nisshin OilliO Group, Ltd. (Tokyo, Japan). 9*c*,11*c*-CLA, 9*c*,11*t*-CLA, 9*t*,11*t*-CLA and 10*t*,12*c*-CLA isomers were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). 1,2-Diheptadecanoyl-*sn*-glycero-3-phosphocholine (DHPC) used as an internal standard was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Bovine serum albumin was purchased from MERCK (Darmstadt, Germany). γ-Globulin from bovine blood was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Casein from milk and gliadin were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and MP Biomedicals, LLC (Eschwege, Germany), respectively. All solvents and other chemicals used in this study were at least of analytical grade.

2.2. Esterification

Typical reaction mixture for synthesis of CLA-PC was LPC 11 mg, CLA 18 mg, PLA_2 3.3×10^4 U, glycerol 550 mg, water or formamide 50 μL and $CaCl_2$ 0.3 μmol. Synthetic reaction of CLA-PC was carried out at 37 °C, 50–200 rpm in the dark. The 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 Section 2.2 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 system was consisted of gas chromatograph (Shimadzu, GC-14B, Kyoto, Japan.) equipped with flame ionization detector and fused silica capillary column, Omegawax 320 (30 m × 0.32 mm i.d.) (Supelco Inc., Bellefonte, PA, USA). Injector, column and detector temperature were 250, 200 and 260 °C, respectively. Helium gas was used as carrier gas and its pressure was adjusted to 50 kPa.

2.5. Calculation of CLA-PC yield

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

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

For studies using purified 9*c*,11*t*-CLA, 10*t*,12*c*-CLA, 9*t*,11*t*-CLA and 9*c*,11*c*-CLA isomers, the yield of synthesized CLA-PC was estimated by GC analysis using DHPC as internal standard. That is, an adequate amount of DHPC solution (0.1 mg/mL chloroform) was added to lipid fraction separated from reaction mixture. The lipid fraction was applied to silica gel TLC plate with fluorescence dye (Silica gel 60 F_{254}, 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 Section 2.4. The yield of synthesized CLA-PC was calculated from the amount of total fatty acid versus heptadecanoic acid from DHPC.

3. Results and discussions

3.1. Synthesis of CLA-PC

We attempted the synthesis of CLA-PC through esterification of LPC with CLA by PLA_2. 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,

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

(wt.%)	LPC	CLA-mixture	CLA-PC
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*c*,11*t*-CLA	–	33.1	13.9
10*t*,12*c*-CLA	–	34.0	15.1

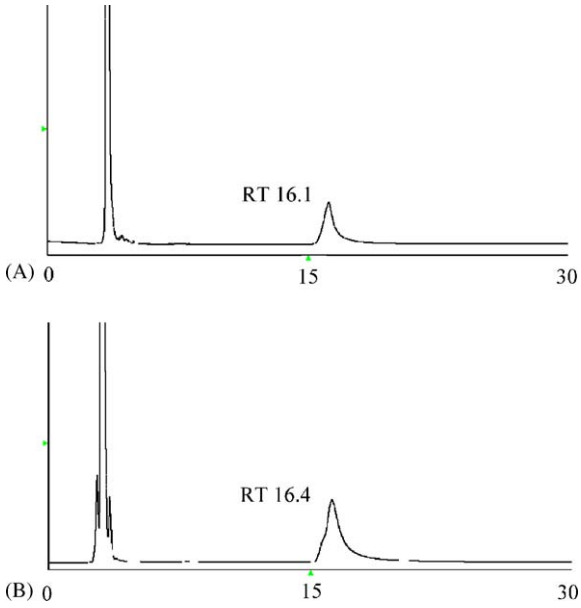

Fig. 1. HPLC chromatogram: (A) PC standard (dioleoylphosphatidylcholine); (B) reaction product.

respectively. These data indicate the synthesis of CLA-PC by PLA_2.

3.2. Effect of formamide on CLA-PC synthesis by PLA_2

Esterification of LPC with CLA mediated by PLA_2 was conducted under low water conditions (Fig. 2). However, the yield of CLA-PC was only 2.1 mol% under the reaction conditions of 11 mg LPC, 18 mg CLA, 3.3×10^4 U PLA_2, 550 mg glycerol, 0.3 μmol $CaCl_2$ and 50 μL water at 37 °C for 48 h. In contrast, addition of formamide in place of water dramatically increased the yield of CLA-PC to 45.8 mol%.

Formamide is known as a "water mimic" that activates enzymes instead of water because of its high dielectric constant and ability to form multiple hydrogen bonds with protein [14,15]. In our previous reports, we showed that formamide is an effective polar solvent in synthesizing PC containing polyunsaturated fatty acids (PUFA) by PLA_2 [13]. That is, formamide promoted the esterification of LPC with PUFA by PLA_2 and suppressed the hydrolysis of PUFA-PC. In the synthesis of CLA-PC, it was suggested that formamide activated PLA_2 and considerably improved the yield.

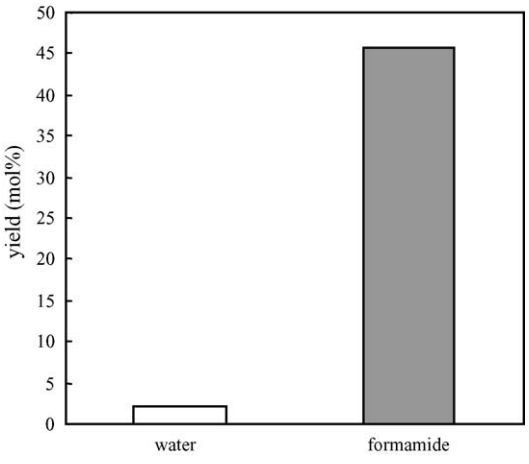

Fig. 2. Effect of formamide on CLA-PC synthesis by PLA_2. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture (9*c*,11*t*-CLA 33.1%, 10*t*,12*c*-CLA 34.0%), 3.3×10^4 U PLA_2, 550 mg glycerol, 0.3 μmol $CaCl_2$, and 50 μL water or 50 μL formamide. The reaction was conducted at 37 °C for 48 h.

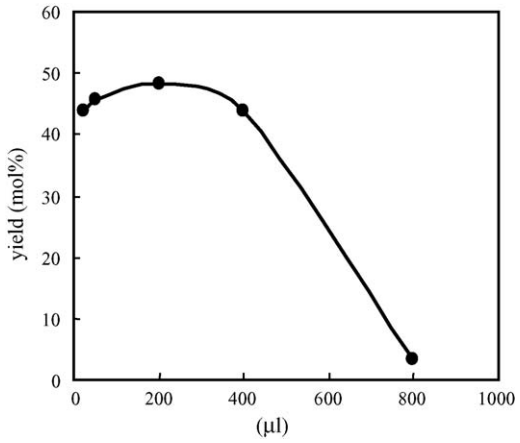

Fig. 3. Optimal amount of formamide for CLA-PC synthesis by PLA_2. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 3.3×10^4 U PLA_2, 550 mg glycerol, 0.3 μmol $CaCl_2$, and 25–800 μL formamide. The reaction was conducted at 37 °C for 48 h.

3.3. Optimal amount of formamide

We examined the optimal amount of formamide for CLA-PC synthesis. The yield of CLA-PC increased with the amount of formamide and reached a maximum of 48.2 mol% at 200 μL of formamide. However, formamide addition over 200 μL decreased the CLA-PC yield (Fig. 3). These data indicate that the optimal amount of formamide is 200 μL for the reaction mixture of 11 mg LPC, 18 mg CLA, 3.3×10^4 U PLA_2, 550 mg glycerol, and 0.3 μmol $CaCl_2$. In the reaction system without formamide, the CLA-PC yield was 22 mol% (data not shown). This may be because glycerol, which was used to disperse the substrate in the reaction system, also plays a role as water mimic, as previously reported [14].

3.4. Optimal amounts of PLA_2, CLA and glycerol

To estimate the optimal amount of PLA_2, the synthesis reaction was conducted in the range of 0.275×10^4 to 5.5×10^4 U (Fig. 4). The yield of CLA-PC increased as the amount of PLA_2 increased and reached a plateau at 0.55×10^4 U. We then examined the effect of the amount of substrate CLA on CLA-PC synthesis by PLA_2. As with PLA_2, the yield of CLA-PC increased dose-dependently up to 72 mg CLA in the reaction system (Fig. 5).

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

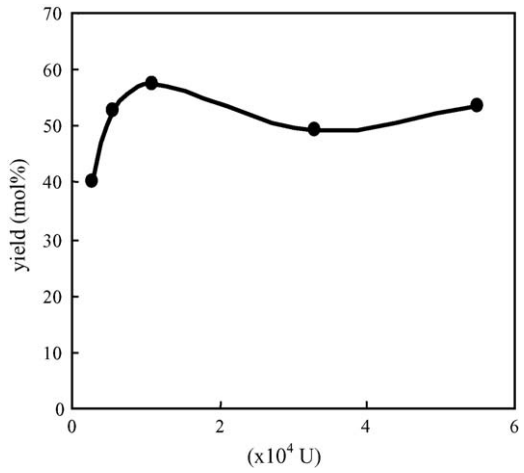

Fig. 4. Effect of the amount of PLA_2 on CLA-PC synthesis by PLA_2. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 50 μL formamide, 0.3 μmol $CaCl_2$, and (0.275–5.5) × 10^4 U PLA_2. The reaction was conducted at 37 °C for 48 h.

3.5. Effect of protein addition

Regulation of the water content around PLA_2 is crucial for CLA-PC synthesis in non-aqueous media because water is also produced by the proceeding esterification even if no water is added to the reaction system. In previous studies, regulation of water content in reaction system by water activity control and decompression has been attempted in PC synthesis by PLA_2 [16,17]. On the other hand, some proteins are known to have hygroscopicity [18]. We therefore investigated the effect of protein on synthesis of CLA-PC (Fig. 6). The addition of albumin, γ-globulin, casein and gliagin increased the CLA-PC yield compared to that without proteins, except for PLA_2 (Fig. 6A). Furthermore, we examined the optimal amount of albumin for CLA-PC synthesis. As shown in Fig. 6B, the addition of albumin in the range of 0.5–10 mg was effective at increasing the yield of CLA-PC after 48 h. In particular, the addition of 1 mg of albumin increased the yield of CLA-PC to 59 mol%. However, the

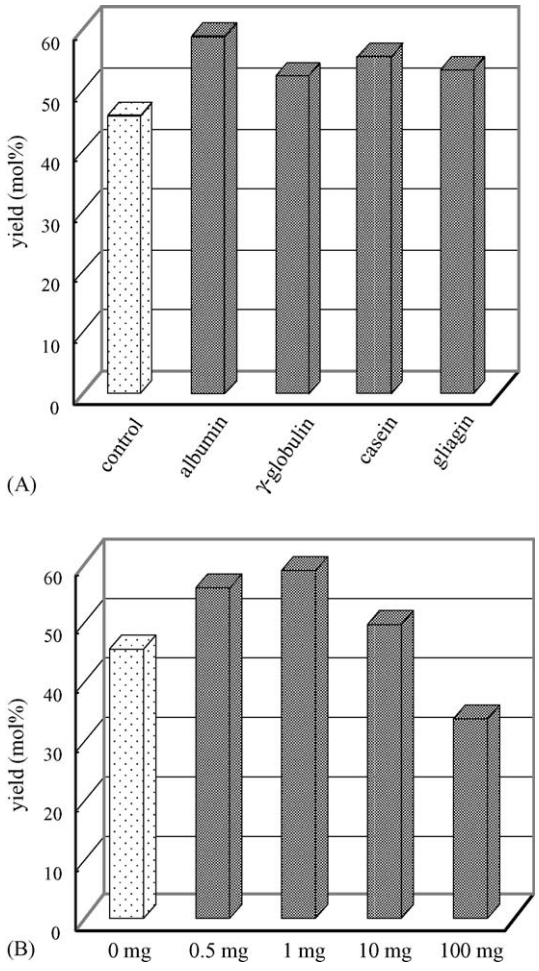

Fig. 6. Effect of protein addition on CLA-PC synthesis by PLA_2. (A) Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3 × 10^4 U PLA_2, 50 μL formamide, 0.3 μmol $CaCl_2$, and 1 mg protein. The reaction was conducted at 37 °C for 48 h. (B) Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3 × 10^4 U PLA_2, 50 μL formamide, 0.3 μmol $CaCl_2$, and 0–100 mg albumin. The reaction was conducted at 37 °C for 48 h.

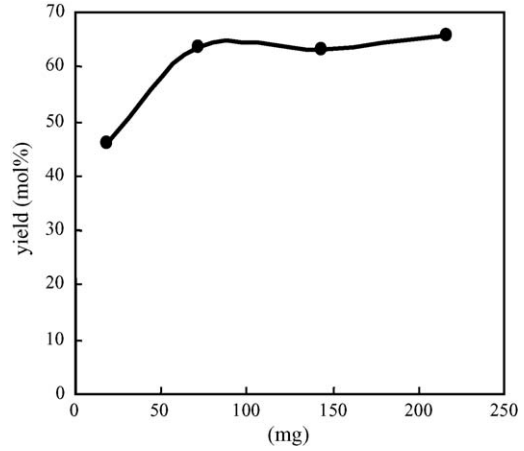

Fig. 5. Effect of the amount of CLA on CLA-PC synthesis by PLA_2. Reaction mixture: 11 mg LPC, 18–216 mg CLA-mixture, 3.3 × 10^4 U PLA_2, 550 mg glycerol, 50 μL formamide, 0.3 μmol $CaCl_2$. The reaction was conducted at 37 °C for 48 h.

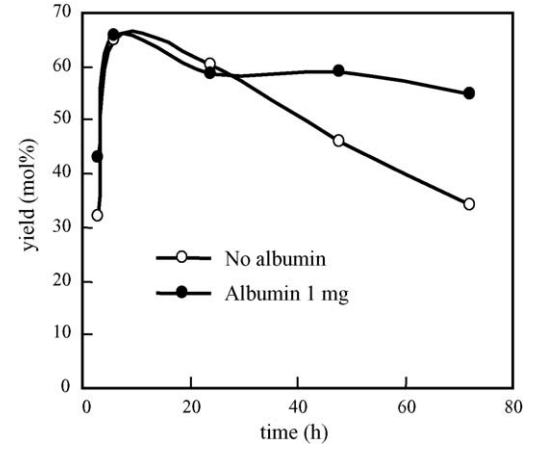

Fig. 7. Time course of CLA-PC synthesis by PLA_2 in the presence of albumin. Reaction mixture: 11 mg LPC, 18 mg CLA-mixture, 550 mg glycerol, 3.3 × 10^4 U PLA_2, 50 μL formamide, 0.3 μmol $CaCl_2$, and 1 mg albumin. The reaction was conducted at 37 °C for 48 h.

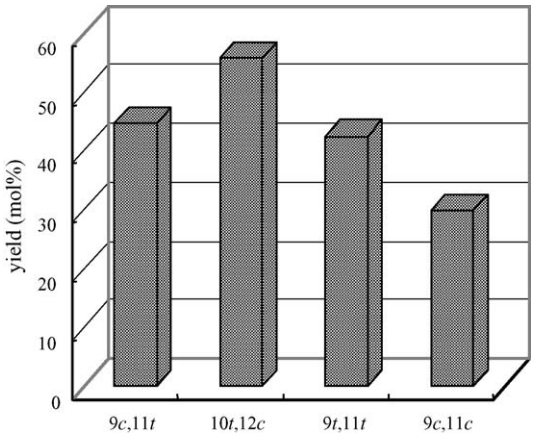

Fig. 8. Substrate specificity of PLA_2 for CLA isomers in the esterification of LPC. Reaction mixture: 11 mg LPC, 18 mg CLA isomer, 3.3×10^4 U PLA_2, 550 mg glycerol, 50 μL formamide, and 0.3 μmol $CaCl_2$. The reaction was conducted at 37 °C for 48 h.

CLA-PC yield decreased by the addition of 100 mg of albumin. This may be because of the high viscosity and a decrease in the water content around PLA_2 due to water absorption by the albumin.

We measured the time course of CLA-PC synthesis by PLA_2 (Fig. 7). The yield of CLA-PC rapidly increased, reaching 65 mol% after 6 h. However, synthesis of CLA-PC gradually decreased after 6 h in the reaction system without albumin. In contrast, the CLA-PC yield in the reaction system with albumin was maintained at approximately 60 mol% even after 6 h. This suggested that water absorbed from atmosphere might shift the reaction equilibrium to hydrolysis in the later stage of the reaction, resulting in a decrease of CLA-PC. Albumin may be able to control the water activity in the reaction system. This study indicated, for the first time, that addition of proteins is effective to maintain CLA-PC yield at a high level.

3.6. Synthesis of CLA-PC using CLA isomers

CLA has several isomers such as 9*c*,11*t*-CLA, 10*t*,12*c*-CLA, 9*t*,11*t*-CLA and 9*c*,11*c*-CLA. In addition, physiological activities of CLA, such as the anti-obesity effect [1], anti-hypertensive effect [19], and anti-cancer effect [20], are known to be different among isomers. We therefore examined the substrate specificity of PLA_2 for esterification using commercially available CLA isomers, 9*c*,11*t*-CLA, 10*t*,12*c*-CLA, 9*t*,11*t*-CLA and 9*c*,11*c*-CLA (Fig. 8).

The reaction yield was the highest for 10*t*,12*c*-CLA and reached 55.9 mol% after 48 h reaction. Both 9*c*,11*t*-CLA and 9*t*,11*t*-CLA, PLA_2 exhibited the same specificity. On the other hand, of the four CLA isomers used in this study, 9*c*,11*c*-CLA was found to be unsuitable for CLA-PC synthesis by PLA_2. These results indicate that porcine pancreatic PLA_2 has different substrate specificity for CLA isomers in the esterification of LPC.

4. Conclusion

This study clarified that esterification of LPC with CLA by PLA_2 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, PLA_2 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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