Advances in Engineering Research, volume 172
4th International Conference on Food and Agriculture Resources (FANRes 2018)

Phospholipase A_2-Mediated Preparation of Phosphatidylcholine Containing Ricinoleic Acid and its Anti-inflammatory Effect *in vitro*

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Abstract— **Ricinoleic acid (RA) is a type of fatty acid found in castor oil and has been known to have anti-inflammatory effects**
Phospholipids are useful functional compounds in food/medical fields due to their amphiphilic property and biocompatibility. The purpose of this study was to prepare phospholipid containing RA and to evaluate the compound's anti-inflammatory activity *in vitro*. Lyso-phosphatidylcholine (LPC) from egg yolk was subjected to phospholipase A_2-mediated esterification for the preparation of phosphatidylcholine containing RA (RA-PC). The prepared RA-PC was then evaluated for its anti-inflammatory activity against the murine macrophage-like cell line RAW264.7. Using glycerol as solvent and formamide as water mimic, RA-PC was successfully prepared. Upon optimizing the molar ratio (RA/LPC), the amount of glycerol, and the reaction time, a maximum yield of 57.5 mol% was obtained. A dose dependent decrease in the expression levels of IL-6 and IL-1β was observed upon addition of RA-PC into the culture medium of RAW264.7. Down regulation of gene expression was higher for RA-PC than for RA or Soy-PC, which suggests that the anti-inflammatory effects of RA were improved following phosphatidylation. Our data suggest that RA-PC is a potential compound for use as an anti-inflammatory agent.

Keywords—anti-inflammatory, phospholipase A_2, ricinoleic acid

I. INTRODUCTION

Ricinoleic acid (RA) is the main fatty acid found in castor oil. It typically has a hydroxyl group at the C12 position (12OH-C18:1 n-9, Fig. 1). Although RA is not used in food materials, it has generated interest for its physiological functions as a laxative, labor inducing agent, and an anti-inflammatory agent [1] [2] [3]. It is also used as one of the raw materials in RipStick creams and pomade for its moderate solubility in ethanol and acetic acid resulting in the appropriate viscosity. However, quality improvement is necessary owing to occasional cases of contact dermatitis[4].

Phospholipids, especially glycero-phospholipids, are desirable substrates for use as physiological compounds in various fields, such as the food industry or the medical industry. This is because of the phospholipids' amphiphilic properties and biocompatibility. These properties allow for the use of phospholipids as food emulsifiers or as raw materials for liposomes, which are used in drug delivery systems [5] [6]. Phosphatidylated RA can be an appealing physiologically active compound that has functions of both RA and phospholipids.

Chemical or enzymatic methods for the preparation of phosphatidylated RA are available. Borsotti et al. reported on the synthesis of phosphatidylated RA (phosphatidylcholine containing RA at *sn*-2 position, RA-PC) using a chemical method. However, the method requires several toxic compounds and involves a complex reaction pathway, which results in only a 10.3% yield [7]. Alternatively, the enzymatic method proceeds with the reaction in simple and mild conditions with no harmful compounds.

Vijeeta et al. reported on the synthesis of RA-PC from soya and egg-PC through phospholipase A1-mediated esterification with a yield of only 10%[8]. This method utilized the *sn*-1 position for the binding of RA. Using phospholipase A_2 (PLA_2), it is possible to combine the desired fatty acid into the glycerophospholipids at the *sn*-2 position [9, 10]. Hosokawa reported the synthesis of PC containing docosahexaenoic acid at the *sn*-2 position through esterification of egg yolk lyso-PC and docosahexaenoic acid mediated by PLA_2 [9]. Our team also reported the synthesis of PC containing conjugated linoleic acid at the *sn*-2 position in the same way [10]. However, the synthesis of PC containing RA at the *sn*-2 position has not yet been reported. In this study, we aimed to prepare RA-PC mediated by PLA_2 (Figure 1) and to evaluate its anti-inflammatory effect in vitro.

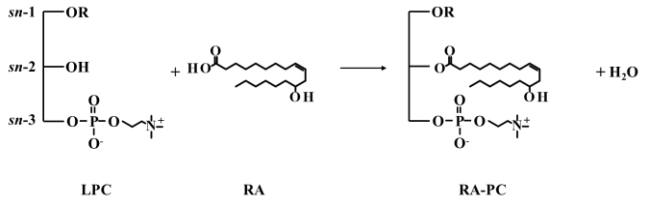

Figure 1. Reaction scheme of PLA2-mediated esterification of LPC and RA

II. METHODS

A. Materials

LPC from egg yolk and RA (>80%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Industrial PLA_2 (Lecitase 10L) from porcine pancreas was obtained from Novozymes A/S (Bagsværd, Denmark). PLA_2 was used after dialysis of Lecitase 10L using a cellulose tube in distilled water, followed by freeze-drying. All solvents and other chemicals used in this study were analytical grade.

B. Enzymatic reaction

The reaction mixture used for RA-PC preparation was: 0.02 mmol of LPC (11 mg), 0.3 mmol of RA (90 mg), 550 mg of glycerol, 3.3 × 104 U of PLA_2 (6 mg), and 50 μL formamide containing 0.3 μmol of $CaCl_2$. Reaction was allowed to proceed at 40°C, 900 rpm, in the dark. The reaction was then terminated though the addition of methanol. Afterwards, chloroform and water were added to the reaction mixture to a final ratio of chloroform/methanol/water = 10:5:3 (v/v/v). The lipid fraction, which includes the synthesized RA-PC, the substrate LPC, and the substrate RA, was obtained from the chloroform layer. The reaction mixture was subjected to thin layer chromatography and revealed a novel spot that had the same Rf value as the PC standard. Detection was done using both I_2 vapor and Dittmer reagent. Confirmation of RA content in the PC spot was done through gas chromatography (data not shown).

C. Calculation of reaction yield

The yield of RA-PC prepared from the reaction was measured through high performance liquid chromatography (HPLC). The chloroform layer from the reaction mixture was introduced into the HPLC system which consists of a Waters 2695 Separations module and a reflective detector model 133 (GILSON). An Inert SIL 10 (4.6 × 250 mm, 5 μm, GL Science) column was used. Samples were then eluted using an isocratic elution of the mobile phase with a ratio of acetonitrile/methanol/sulfuric acid = 100:10:0.05 (v/v/v). The flow rate was maintained at 1.0 mL/min, while the column temperature was maintained at 40°C. Molar ratio (synthesized RA-PC/substrate LPC) was calculated from the calibration curve as follows:

$$y = 1.9616x - 0.1047 \ (R^2 = 0.9998) \quad (1)$$

The variable "y" stands for the area ratio (synthesized RA-PC/substrate LPC) in the reaction mixture, while the variable "x" stands for the molar ratio (synthesized RA-PC/substrate LPC). Reaction yield of RA-PC was then calculated following the equation below.

$$\text{Yield (mol\%)} = \frac{\text{RA-PC (mol/LPC (mol)) in the reaction mixture}}{\text{[RA-PC (mol/LPC (mol)) in the reaction mixture + 1 (LPC (mol))]}} \times 100 \quad (2)$$

D. Cell culture

Murine macrophage-like cell line RAW264.7 was purchased from DS Pharma Biomedical (Suita, Osaka, Japan). RAW264.7 cells (5×10^4 cells/well) were pre-incubated in 24-well plates with 1 mL RPMI 1640 containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. RA-PC, Soy-PC (H. Holstein), and RA were then added into the culture medium and the cells were incubated for 24 h. Each sample was added into the culture medium as an ethanolic solution. Final concentration of ethanol was adjusted to 0.1% in the culture medium without cytotoxicity. Afterwards, cell inflammation was induced using lipopolysaccharide (LPS, final concentration of 0.1 μg/mL) for 6 h in the presence of the sample.

E. Quantitative real-time RT-PCR

After incubation of the sample with LPS, the RAW264.7 cells were washed thrice with PBS. Total RNA was extracted from the cells using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer's protocol. Afterwards, cDNA was synthesized from total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems Japan Ltd, Tokyo, Japan). Quantitative real time RT-PCR was then performed using the ABI Prism 7500 (Applied Biosystems Japan Ltd, Tokyo, Japan). Cycling conditions for PCR were as follows; 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. PCR primers and TaqMan® probes were obtained from TaqMan® Gene Expression Assays (Applied Biosystems Japan Ltd, Tokyo, Japan); IL-6: Mm00446190_ml, IL-1β: Mm00434228_ml, 18S: Mm02601777_g1.

F. Statistical analysis

For RA-PC preparation: All values are expressed as mean ± SD (n = 3). Statistical differences were determined by the Scheffe's F test at $P < 0.05$. For evaluation of anti-inflammatory effect: All values are expressed as mean ± SE (n = 3). Statistical differences were determined by the Tukey's F test at $P < 0.05$ or $P < 0.01$.

III. RESULTS AND DISCUSSION

A. Effect of the molar ratio of RA/LPC on the RA-PC preparation

The effect of the molar ratio of RA/LPC on the RA-PC preparation was first investigated with reaction mixture of 0.02 mmol of LPC, 0.02~0.40 mmol of RA, 550 mg of glycerol, 3.3 × 104 U of PLA2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h.

Initially, RP-PC yield increased with the increase in molar ratio of RA/LPC. This may be due to the increased contact frequency in this range of molar ratios (Figure 2). However, after the yield reached a peak of 40.5 mol% at a molar ratio of 10, the yield began to decrease. At a molar ratio of 20, a yield of only 20.7 mol% was obtained.

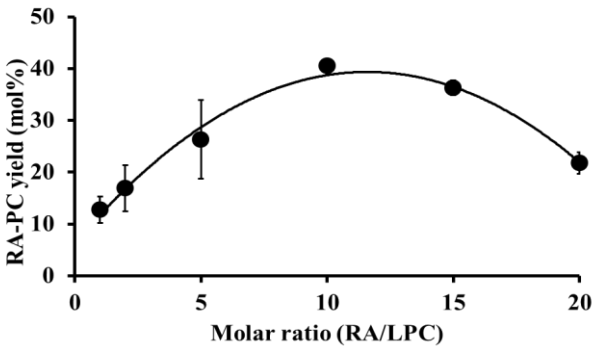

Figure 2. Effect of molar ratio (RA/LPC) on the RA-PC preparation

In the PLA_2-mediated esterification of LPC and oleic acid, excess amount of oleic acid caused high viscosity and high polarity in the reaction mixture, and resulted in lower reaction rate, but did not affect the reaction yield [**11**]. In our previous study on the preparation of PC containing conjugated fatty acids, it was also reported that the yield increased with the increase in molar ratio of conjugated fatty acid/LPC (up to a molar ratio of 40 [**10**]. Therefore, this result was typical for the reaction using RA as acyl donor. In the current study, the high polarity of RA, brought about by its hydroxyl group, might cause the deactivation of PLA_2. As such, the optimal molar ratio of RA/LPC was determined to be 10.

B. Effect of the amount of glycerol on the RA-PC preparation

The effect of the amount of glycerol on the RA-PC preparation was then investigated with the reaction mixture of 0.02 mmol of LPC, 0.20 mmol of RA, 225~2200 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 24 h.

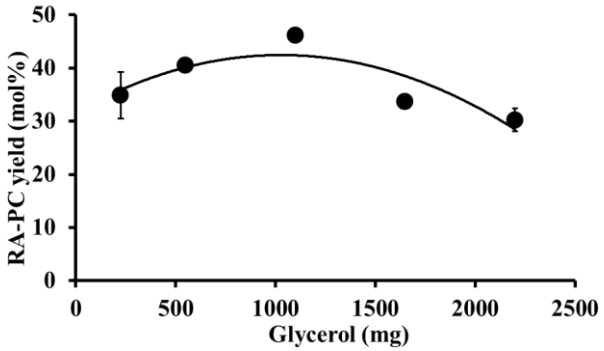

Figure 3. Effect of the amount of glycerol on the RA-PC preparation

The yield increased with the addition of glycerol up to 1100 mg (Figure 3). A maximum yield of 46.1 mol% was obtained; however, a decrease in yield was observed with the addition of glycerol beyond 1100 mg. The increase in yield in the range of 225~1100 mg of glycerol is attributed to the increase in the dispersibility of reaction components such as RA, LPC, and PLA_2. However, in the range of 1100~2200 mg of glycerol, the dilution effect will be high, resulting in a low yield. Therefore, optimal amount of glycerol was determined to be 1100 mg.

C. Effect of the reaction time on the RA-PC preparation

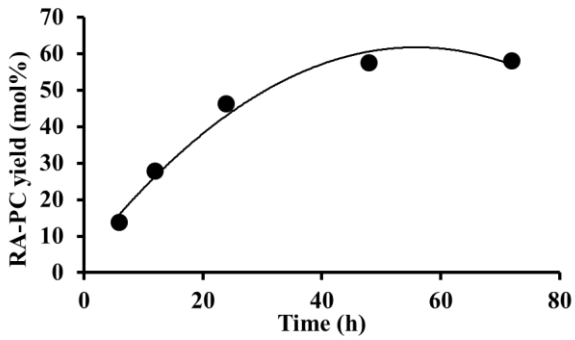

Figure 4. Effect of reaction time on the RA-PC preparation

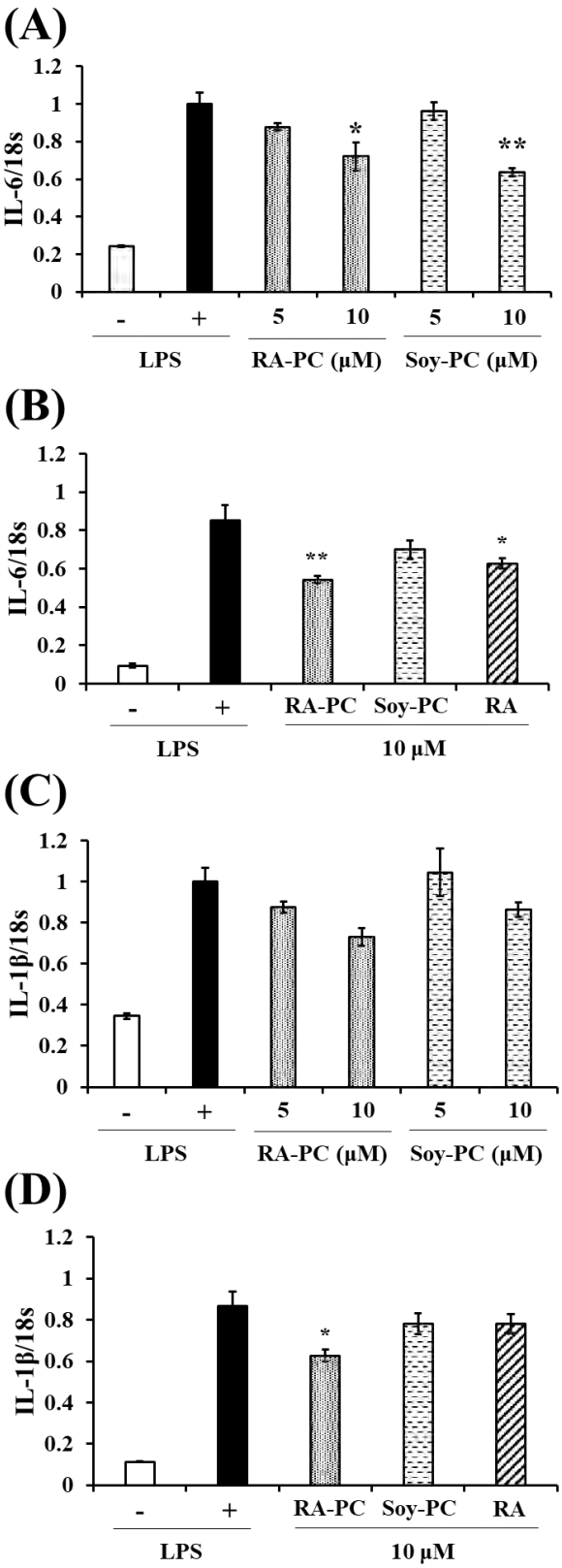

Figure 5. Anti-inflammatory effects of RA-PC on RAW264.7

Finally, effect of the reaction time on the RA-PC preparation was investigated with reaction mixture of 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, $3.3 \times$

10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$, with a reaction time varying from 6~72 h.

The yield increased with the additional reaction time, but reached a plateau at 48 h, where a maximum yield of 57.5 mol% was obtained (Figure 4). The use of linoleic acid (C18:2 n-6) as the fatty acid substrate for this reaction resulted in a yield of 43.4 mol%., suggesting that the hydroxyl group of RA at C12 position is not a steric hindrance to this reaction.

Therefore, the optimum reaction condition for RA-PC synthesis was determined to be 0.02 mmol of LPC, 0.20 mmol of RA, 1100 mg of glycerol, 3.3×10^4 U of PLA_2, and 50 μL formamide containing 0.3 μmol of $CaCl_2$ for a reaction time of 48 h.

D. Anti-inflammatory effect of RA-PC

Anti-inflammatory effects of RA-PC on RAW264.7 cells were then investigated through the expression levels of IL-6 and IL-1β mRNA. These are inflammatory cytokines that can be used as indicators of inflammation. (Figure 5).

Expression of IL-6 and IL-1β mRNA, induced by LPS stimulation, was observed to have a dose-dependent suppression upon addition of RA-PC and Soy-PC (Figure 5A, C). Comparing the expression of IL-6 mRNA in the presence of RA-PC, Soy-PC, and RA, the observed suppression order was RA-PC ($P < 0.01$), RA ($P < 0.05$), and Soy-PC (no significance) (Figure 5B). A similar result was obtained from the expression of IL-1β mRNA (Figure 5D). This result suggested that anti-inflammatory effects of RA increased after phosphatidylation.

Physiological functions of several bioactive compounds are often improved through phosphatidylation **[12] [13] [14].** For example, cytotoxicity of phosphatidylated genipin was found to be higher against some cancer cell lines than that of genipin [13]. Phosphatidylated terpenes, such as geraniol and farnesol, showed stronger anti-proliferative effects than their non-phosphatidylated counterparts [**14**]. These reports found that the cause for improved physiological functions was the increase in cellular intake of the phosphatidylated compounds brought about by the amphiphilic property of phospholipids. The same mechanism may explain the improved anti-inflammatory effect of RA after phosphatidylation.

IV. CONCLUSION

RA-PC was successfully prepared with optimal reaction mixture: 0.02 mmol of lpc, 0.20 mmol of ra, 1100 mg of glycerol, 3.3 × 104 u of pla2, and 50 μl formamide containing 0.3 μmol of cacl2, for a reaction time of 48 h. This resulted in a yield of 57.5 mol%. The anti-inflammatory effect of ra-pc was found to be higher than that of ra and soy-pc, suggesting that ra-pc is a potential anti-inflammatory agent.

ACKNOWLEDGMENT

We would like to thank Editage (www.editage.jp) for English language editing.

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