Effect of Molecular Form of Conjugated Linoleic Acid on Oxidative Stability: Comparison of Triacylglycerol and Phosphatidylcholine Form

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Abstract: The health benefits of conjugated linoleic acid (CLA), a functional lipid with anti-cancer, anti-obesity, and hypotensive activity, have garnered increasing attention. The current study was conducted to determine the oxidative stability of CLA in the form of triacylglycerol (CLA-TAG) and phosphatidylcholine (CLA-PC) at the sn-2 position. Oxidation was performed at 30°C or 40°C in the dark. Hydroperoxides, as the primary oxidation products, were analyzed using diphenyl-1-pyrenylphosphine. Thiobarbituric acid reactive substances (TBARS) and volatile compounds were monitored as secondary oxidation products. The results suggest that CLA-PC was more stable against oxidation than CLA-TAG from the perspective of suppression of the generation of hydroperoxides and TBARS. However, CLA-PC produced more volatile compounds than CLA-TAG. We suggest that choline was released during the oxidation of CLA-PC, and acted as an antioxidant. The ensuing reaction between choline and hydroperoxide induced the generation of volatile compounds such as pentanal, hexanal, and heptanal.

Key words: oxidation stability, conjugated linoleic acid, triacylglycerol, phosphatidylcholine

1 Introduction

Conjugated linoleic acid (CLA) refers to cis/trans conjugated isomers of linoleic acid. The bioactivity of CLA, as an anti-cancer component of beef, was first reported in 1987. Since then, various physiological functions of CLAs, such as anti-cancer activity, reducing the risk of cardiovascular disease, anti-obesity, hypotensive activity, improving muscle metabolism, improving bone health, and modulating immune and inflammatory responses, have been reported and the health impact of CLAs continues to attract attention.

However, the availability of conjugated carbon double bonds in the CLA molecule leads to facile oxidation of this fatty acid. For example, van den Berg et al. reported that the oxidative stability of the fatty acid residue of CLA was lower than that of its unconjugated form (linoleic acid) when exposed to air at room temperature. In an experiment conducted by Zang and Chen, it was found that the free fatty acid (FFA) and triacylglycerol (TAG) form of CLA were both as highly unstable as docosahexaenoic acid (DHA) in the bulk phase at 90°C and were more rapidly oxidized than linoleic acid, linolenic acid, and arachidonic acid. It is known that the oxidative stability of lipids is affected not only by their fatty acid compositions, but also by their molecular species and lipid form. For example, in the TAG form, polyunsaturated fatty acids (PUFAs), such as DHA and eicosapentaenoic acid (EPA), substituted at the sn-2 position, are more stable than those substituted at the sn-1 (3) position. A similar conclusion was reached for TAG containing conjugated linolenic acid based on conductometric determination under oxidative conditions (90°C and air flow of 20 L/h). However, few studies have compared the oxidative stability of the TAG form and phospholipid form of unsaturated fatty acids. Of the few reports, Grandois et al. reported that the phosphatidylcholine (PC) form of oleic acid and linoleic acid is more resistant to oxidative degradation than the TAG form when oxidized under heating conditions (50-175°C).

To date, no comparative analysis of the oxidative stability of CLA in the TAG and PC form, with CLA substituted at the sn-2 position (CLA-TAG and CLA-PC), has been documented. The objective of this study is to reveal the effects of the molecular form of CLA, with different lipid forms.
TAG and PC), on the oxidative stability.

2 Experimental

2.1 Materials

Two commercially available CLA samples (CLA Gold Gym™ as the FFA form and CLA Jarrow™ as the TAG form) were used as the substrate for preparation of CLA-TAG and CLA-PC. These samples do not contain any additional compounds, only oil and tocopherols. The tocopherols in the samples were removed using activated charcoal powder (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and the sample integrity was checked by high performance liquid chromatography, as reported before. Palmitic acid from FUJIFILM Wako Pure Chemical Corporation and Lipozyme® RM-IM from Novozymes A/S ( Bagsvaerd, Denmark) were used in the preparation of CLA-TAG. CLA-PC was prepared by using 1,α-lysophosphatidylcholine (LPC) from egg yolk and glycerol, which were purchased from Kewpie Corporation (Tokyo, Japan) and FUJIFILM Wako Pure Chemical Corporation, respectively. Phospholipase A2 (PLA2) (Lecitase 10L) from porcine pancreas was purchased from Novozymes A/S. PLA2 was used after dialysis of Lecitase 10L against distilled water in a cellulose tube, followed by freeze-drying. All solvents and other chemicals used were at least of analytical grade.

2.2 Preparation of CLA-TAG

CLA-TAG was prepared by acidolysis using Lipozyme® RM-IM. One gram (≈1.1 mmol) of the TAG form of CLA and 850 mg (≈1.1 mmol) of palmitic acid were dissolved in 4 mL of iso-octane, followed by the addition of 0.2 g (30 IUN) of Lipozyme® RM-IM. The mixture was incubated in the dark at 60°C, and agitated at 600 rpm for 48 h. To terminate the reaction, the enzyme was filtered off and the filtrate was removed by evaporation. This reaction mixture was applied to a Florisil® column for chromatographic separation to obtain the target CLA-TAG fraction, as previously reported.

2.3 Preparation of CLA-PC

CLA-PC was prepared by esterification using PLA2, as previously reported. Eleven milligrams (≈0.02 mmol) of LPC and 60 mg (≈0.2 mmol) of the FFA form of CLA were mixed with 1,100 mg of glycerol. Thereafter, 6 mg (≈3.3 × 10⁴ U) of PLA2 and 50 μL of formamide containing 0.3 μmol CaCl₂ were added. The mixture was incubated in the dark at 37°C with agitation at 600 rpm for 48 h. To terminate the reaction, 2 mL of methanol was added. Thereafter, chloroform-methanol-water (1:1:1, v/v/v) and the chloroform layer was evaporated to obtain the reaction mixture. This mixture was applied to a silica gel column for chromatographic separation with chloroform and methanol as the mobile phase to obtain the polar lipids, followed by preparative thin layer chromatography to obtain the target CLA-PC fraction, using chloroform-methanol-water (65:25:4, v/v/v) as the developing solvent.

2.4 Analysis

2.4.1 Fatty acid composition

The fatty acids in the samples containing CLA were methyl-esterified using sodium methoxide, while LPC and palmitic acid treated by following Jham’s method. The samples were subjected to gas chromatography using a GC-17A (Shimadzu Corporation, Kyoto, Japan) instrument. This instrument was equipped with a flame ionization detector and a fused silica capillary column (DB-WAX; 60 m × 0.25 mm, 0.25 μm; Agilent Techn., USA). The temperature of both the injector and detector was 250°C. Helium at 80 kPa was used as the carrier gas. The column temperature was 190°C.

2.4.2 Analysis of primary oxidation products

The primary oxidation products were analyzed by quantifying the hydroperoxide in the samples by using diphenyl-1-pyrenylphosphine (DPPP), as described by Santas et al. with slight modification. The samples (0.3 mg) were oxidized in small glass vials at 40°C in the dark; the oxidized samples were then dissolved in 50 μL of 4 mM butylated hydroxytoluene (BHT) in ethanol, followed by the addition of 100 μL of 100 μM DPPP in ethanol containing 4 mM BHT. The mixture was incubated at 60°C for 60 min, then cooled in an ice bath for 10 min, and kept at room temperature for 5 min. The fluorescence intensity (Ex. 352 nm, Em. 380 nm) of the solution was measured using a micro-plate reader (Varioskan, Thermo Fischer Scientific, USA). A standard curve was constructed by using cumene hydroperoxide, for quantification, where the data are as expressed as μmol/mg lipid.

2.4.3 Analysis of aldehydes as secondary oxidation products

The aldehydes, as secondary oxidation products, were analyzed by quantifying the thiobarbituric acid reactive substances (TBARS), as described by Buege and Aust with slight modification. The samples (5 mg) were oxidized in a 10 mL glass test tube at 40°C in the dark, and the oxidized samples were dissolved with 2 mL of ethanol, followed by the addition of 2 mL of thiobarbituric acid (TBA) solution (0.25N HCl containing 0.015% trichloro acetic acid, 0.1% TBA and 0.01% BHT). The mixture was kept at 100°C for 15 min. The reaction was terminated by cooling in an ice bath for 5 min. After centrifugation (3,000 rpm, 10 min), the absorbance of the supernatant at 532 nm was measured by spectrophotometry (U-2001, Hitachi, Japan). The TBARS(μmol/mg lipid) value was calculated by using Equation (1).
Oxidative Stability of CLA-TAG and CLA-PC

J. Oleo Sci.

2.4.5 Quantification of choline

where the data are expressed as the corresponding authentic compounds for quantification, compounds. A standard curve was constructed by using the NISTII database and authentic compounds. Then the temperature was set to increase at a rate of $7 \degree C/\text{min}$, for 5 min, and then the temperature was set to increase at a rate of $7 \degree C/\text{min}$ for 5 min to reach the final temperature of $240 \degree C$. Helium (140 kPa) was used as the carrier gas. The Peaks were identified by using the NISTII database and authentic compounds. A standard curve was constructed by using the corresponding authentic compounds for quantification, where the data are expressed as $\mu$mol/mol lipid.

2.4.6 Statistical analysis

All experiments were performed in triplicate. The data were expressed as the means ± standard deviations. A significant difference was determined by Scheffe’s test ($p < 0.05$).

3 Results and Discussion

3.1 Fatty acid composition of substrates

The substrates used in this study were CLA Gold Gym™, CLA Jarrow™, LPC, and palmitic acid. The fatty acid composition of these substrates is presented in Table 1. Both CLA Gold Gym™ and CLA Jarrow™ contained CLA in an almost similar amount of approximately 75 wt%, as the sum of two CLA isomers (9c11t and 10t12c). The isomers ratio was the same for both samples. LPC contained only palmitic acid (74.9 ± 0.8 wt%) and stearic acid 25.1 ± 0.1 wt%, suggesting that the sn-1 position of LPC was exclusively composed by saturated fatty acids.

3.2 Preparation of CLA-TAG

To prepare CLA-TAG, CLA Gold Gym™ was transesterified with palmitic acid, catalyzed by Lypozyme® RM-IM. Lypozyme® RM-IM specifically recognizes fatty acids at the sn-1 and sn-3 positions in the TAG structure. The fatty acid composition of the product is presented in Table 2. As shown in Table 2, the concentration of palmitic acid and CLA was 60.6 ± 1.7 % and 31.4 ± 0.7%, respectively. The ratio of palmitic acid to CLA was 2:1, indicating successful transesterification.

| Table 1 | Fatty acid compositions of substrates used in this study (wt %). |
|---------|---------------------------------------------------------------|
| Fatty acid | CLA Jarrow™ | CLA Gold Gym™ | LPC | Palmitic acid |
| C16:0 | 1.9 ± 0.1 | 5.1 ± 0.6 | 74.9 ± 0.8 | 100.0 ± 0.0 |
| C18:0 | 2.5 ± 0.1 | 1.5 ± 0.1 | 25.1 ± 0.1 | – |
| C18:1 | 15.3 ± 0.2 | 12.9 ± 0.6 | – | – |
| C18:2 | 0.6 ± 0.1 | 1.3 ± 0.4 | – | – |
| 9c11t CLA | 37.6 ± 0.4 | 36.6 ± 0.3 | – | – |
| 10t12c CLA | 37.5 ± 0.2 | 36.9 ± 0.7 | – | – |
| n.i. | 4.8 ± 0.5 | 5.7 ± 0.9 | – | – |

n.i.: not identified, –: not detected; data shown is mean values ± SD (n=3)

| Table 2 | Fatty acid compositions of CLA-TAG and CLA-PC used in this study (wt %). |
|---------|---------------------------------------------------------------|
| Fatty acid | CLA-TAG | CLA-PC |
| C16:0 | 60.6 ± 1.7 | 36.1 ± 0.9 |
| C18:0 | – | 12.1 ± 0.4 |
| C18:1 | 7.9 ± 0.2 | 8.1 ± 0.5 |
| 9c11t CLA | 16.0 ± 0.7 | 19.6 ± 0.8 |
| 10t12c CLA | 15.5 ± 0.7 | 16.6 ± 0.6 |
| n.i. | – | 7.5 ± 1.1 |

n.i.: not identified, –: not detected; data shown is mean values ± SD (n=3)
3.3 Preparation of CLA-PC
To prepare CLA-PC, CLA Jarrow™ was esterified with LPC, catalyzed by PLA₂. During this reaction, CLA is incorporated at the sn-2 position of LPC. The fatty acid composition of the product is shown in Table 2. As shown in Table 2, the concentration of palmitic acid and CLA was 36.1 ± 0.9 and 36.2 ± 0.7, respectively. The ratio of palmitic acid to CLA was 1:1, indicating successful esterification.

3.4 Oxidative stability of CLA-TAG and CLA-PC
To investigate the oxidative stability of CLA-TAG and CLA-PC, we monitored the generation of both primary and secondary oxidation products. As primary oxidation products, the hydroperoxides were quantified by using DPPP (2.4.2), and as secondary oxidation products, we measured the TBARS(2.4.3) and volatile compounds (2.4.4). In all oxidation tests, CLA-TAG was in the liquid state, while CLA-PC was semi-solid.

3.4.1 Primary oxidation products of CLA-TAG and CLA-PC
The amount of hydroperoxides generated during the oxidation of CLA-TAG and CLA-PC was monitored by using DPPP; the results are shown in Fig. 1. More hydroperoxides were generated from CLA-TAG than from CLA-PC, even though CLA was bound at the sn-2 position with TAG. After 1 d of oxidation, the amount of hydroperoxide generated from CLA-TAG increased sharply and increased for 4 d, but plateaued thereafter. For CLA-PC, little hydroperoxide was formed until day 8 of oxidation process. The amount of hydroperoxide generated from CLA-TAG at 4 d of oxidation was 144.9 ± 2.5 µmol/mol lipid, which is significantly higher than that generated from CLA-PC (21.1 ± 3.5 µmol/mol lipid). This result indicates that CLA-PC was more stable against oxidation than CLA-TAG in terms of hydroperoxide formation.

3.4.2 Secondary oxidation products of CLA-TAG and CLA-PC
To assess the secondary oxidation products of CLA-TAG and CLA-PC, we monitored the formation of TBARS (Fig. 2) and volatile oxidation products by headspace GC-MS (Fig. 3). The amount of TBARS generated from CLA-TAG increased at the beginning of the reaction and continued to increase up to 4 d of oxidation. The amount of TBARS generated from CLA-TAG at 4 d of oxidation was 0.9 ± 0.1 µmol/mol lipid, while that generated from CLA-PC was 0.4 ± 0.0 µmol/mol lipid, where the value are significantly different. After 4 d of oxidation, the formation of TBARS from CLA-TAG was decreased, which is attributed to be the formation of another secondary or tertiary oxidation product, as described by Kamal-Eldin[18]. For CLA-PC, the production of TBARS increased slowly up to 6 d of oxidation, after which it increased sharply. Similar to the trend for hydroperoxide formation, malondialdehyde (as a secondary oxidation product that is monitored in TBA method) formation from CLA-TAG was greater than from CLA-PC.

On the other hand, only pentanal was detected as a major volatile product of CLA-TAG after 6 d of oxidation, with a concentration of 30.1 ± 2.8 µg/mol lipid (Fig. 3a). However, for CLA-PC, pentanal, hexanal, and heptanal were detected as three major volatile compounds in the same oxidation period (Fig. 3b). The concentration of pentanal, hexanal, and heptanal was 209.9 ± 44.1, 965.4 ± 15.9, and 955.5 ± 39.8 µg/mol lipid, respectively.

Surprisingly, these results show that the PC form of CLA was stable in terms of suppressing primary oxidation, but was unstable in terms of suppressing the generation of volatile compounds. Similar results were reported by Grandois et al.[19] who found that the PL form of oleic acid and linoleic acid (unsaturated fatty acids) was more resistant to oxidative degradation than the TAG form. It has been reported that the side-chain moieties of phospholipids, such as choline, ethanolamine, and serine, can inhibit an increase of the peroxide value by decomposing lipid hydroperoxides into the corresponding hydroxyl lipids[20, 21]. Those authors proposed that intramolecular hydroxymines such as ethanol amine and choline might form bonds with the...
Oxidative Stability of CLA-TAG and CLA-PC

J. Oleo Sci.

Oxygen of hydroperoxide by donating an electron pair to a site that is electron-deficient (Scheme 1). The nucleophiles donating an electron may attack the electron-deficient oxygen, eventually decomposing the peroxides to alcohols. However, choline is a quaternary ammonium cation, not a primary or secondary amine, thus it cannot donate an electron lone pair. Here, we hypothesized that the choline moiety will be released from the PC molecule during oxidation, and acts as a base-catalyst to promote Kornblum-DeLaMare rearrangement on the alkyl hydroxyperoxide to produce alkyl ketone (Scheme 2). This alkyl ketone will be decomposed by reduction by accepting a hydrogen radical, which is generated from another lipid or alkyl hydroperoxide, to olefinic carbon, and finally, corresponding aldehydes such as pentanal, hexanal, and heptanal will be generated (Scheme 2), as indeed observed herein.

To prove that choline is released during oxidation, we quantified the choline released from CLA-PC during oxidation using an Ampilite™ Choline Quantitation Kit. As shown in Fig. 4, the amount of choline increased with the oxidation period. On 7 d of the oxidation period, 72.0 ± 7.3 nmol/mol PC of choline was released. This result supports our hypothesis.

4 Conclusion

We propose that the PC form of CLA is more stable than the TAG form by considering the formation of hydroperoxide and TBARS as decomposition products, because PC has a side-chain moiety, choline, that acts as a self-antioxidant. However, choline also induces the formation of by-products. The by-products generated in this study were volatile oxidation compounds such as pentanal, hexanal,

Fig. 3 Chromatogram of volatile compounds found in (a) CLA-TAG (b) CLA-PC after oxidized at 30°C.

Scheme 1 Decomposition of hydroperoxide proposed by reference 17) and 18).

J. Oleo Sci.
and heptanal.

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Fig. 4 Choline released during oxidation period of CLA-PC after oxidized at 40°C in the dark. Error bars indicate SD (n = 3).
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