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Synthesis of Triglyceride of Conjugated Linoleic Acid (CLA) by Lipozyme

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Abstract

Most fatty acids in food matrices are triglyceride (TG) forms. Conjugated linoleic acid (CLA) produced from linoleic acid by microorganisms or chemicals is a free form. To apply the CLA to food systems, the TG containing CLA (designate CLA-TG) was synthesized by Lipozyme-catalyzed esterification method. An optimum reaction condition for the esterification of free CLA (FCLA) to glycerol by Lipozyme was determined as follows; Lipozyme (50 mg) effectively catalyzed the esterification of CLA (500 mg) to glycerol (1150 mg) dissolved in isooctane (3 ml) in a shaking incubator (200 rpm, 50°C) for 48 hr. Under the reaction condition, the resultant contained 52.4% CLA-TG as well as 31.1% Di-CLA-glycerol (CLA-DG), 7.6% mono-CLA-glycerol (CLA-MG), and 9.0% other CLA (unreacted FCLA plus CLA dimer). These results suggest that the Lipozyme could be a useful enzyme for the production of CLA-TG to be employed in foods.

Key words: conjugated linoleic acid (CLA), glycerol derivatives of CLA, CLA-TG

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for a group of positional (C_9,C_{11} and C_{10},C_{12}) and geometric (*cis,cis*; *cis,trans*; *trans,cis*; and *trans,trans*) isomers of octadecadienoic acid (linoleic acid) with a conjugated double bond system (1,2). It was first identified as comprising an anticarcinogen, principally present in grilled ground beef (1) and later, found in many other food sources, especially dairy products (3-8).

Synthetic CLA, composed of mainly 46% *cis*-9,*trans*-11 CLA and 48% *trans*-10,*cis*-12 CLA, inhibits the carcinogen-induced neoplasia in several animal models (1,9-13). Other beneficial biological activities such as the reduction of atherosclerosis (14), the modulation of immunity (15,16), the stimulation of growth (17) and reduction of body fats (18) were evident.

Food matrices, including animal tissues, milks and cheeses, contain CLA as triglyceride and phospholipid forms (1,3-8). Fats and oils derived from plants and animals contain the triglyceride forms of fatty acids. The free form of CLA (designate FCLA) has a limitation in food uses, due to its oxidative stability and toxicity. Hence, for the special uses, FCLA needs to be esterified to glycerol to form the tri-CLA-glycerol (CLA-TG), which is, in turn, added to foods, fats, and oils. CLA-TG exhibits beneficial effects on the absorption and also exhibits lower toxicity in the guts of animals and humans than FCLA.

The objective of the present research is to investigate reaction conditions for the synthesis of CLA-TG from the FCLA, chemically synthesized from linoleic acid by the alkaline isomerization, and glycerol by Lipozyme.

MATERIALS AND METHODS

Materials

Linoleic acid (99%) was purchased from Doosan Co., (Seoul, Korea). CLA was synthesized from linoleic acid by alkaline isomerization, according to the method described by Kim et al. (19). Lipozyme was obtained from Novonorsk (Netherlands). Glycerol, isooctane, pentane, hexane and ethyl acetate were obtained from Aldrich Chemical Co., (Milwaukee, WI). Aluminum plates (20×20 cm) precoated with silica gel 60 F_{245} were obtained from Merk (Germany). All other chemicals used were ACS grade.

Esterification of CLA with glycerol

Lipozyme-catalyzed esterification of FCLA with glycerol was performed in a shaking incubator (200 rpm, 50°C; Korea Instrument Co., Korea). An appropriate solvent was first selected for the synthesis of CLA-TG by Lipozyme, followed by determining the optimum conditions for the mole ratio of glycerol to CLA, the duration of reaction time, and the amount of Lipozyme. An appropriate mole ratio of glycerol to CLA dissolved in a given solvent was reacted under nitrogen for a period of time. The resultant was filtered through a filter paper, and the residue was washed with the solvent used. Combined filtrates were dried under vacuum for further analysis.

Purification and spectral analysis of CLA derivatives of glycerol

The resultant from Lipozyme-catalyzed esterification of CLA

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with glycerol was dissolved in hexane (2 ml). An aliquots of the hexane solution was applied on the precoated silica gel TLC with a solvent system of hexane:ethylacetate (5:2, v/v). Each fraction separated on the plate was scraped off for the spectral analysis.

UV spectrum of the sample was recorded with a Beckman DU-70 model (USA). IR spectrum was obtained from Hitachi 270-50 (Japan), using a NaCl plate. ^{1}H-NMR and ^{13}C-NMR spectra were obtained from Bruker AW-500 (USA) using $CDCl_3$. EI-MS data was obtained from Jeol JMS-700 (Japan) with ion source of 70 eV.

Quantification of FCLA and CLA derivatives by GC analysis

The samples of FCLA and CLA derivatives were methylesterified with 0.05 N H_2SO_4-catalyzed transesterifiction method described by Kim et al. (19) and then analyzed by GC (Hewlett Packard 5890) equipped with flame ionization detector (FID) and Supelcowax-10 capillary column (60 m × 0.32 mm, i.d., 0.24 μm film thickness). Carrier gas was N_2. Oven temperature was increased from 50°C to 200°C at a rate of 10°C/min. Injector and detector temperatures were set at 240°C and 260°C, respectively.

RESULTS AND DISCUSSION

Separation of CLA-esterified with glycerol

The resultant from Lipozyme-catalyzed esterification of the CLA, synthesized by the method of Kim et al. (19), with glycerol was separated by a precoated TLC, using a hexane: ethylacetate (5:2, v/v) as an eluent. The resultant contained five fractions of A-1 (R_f=0.88), A-2 (R_f=0.78), A-3 (R_f=0.30), A-4 (R_f=0.18) and A-5 (R_f=0.01). The A-2 fraction possesses a similar R_f value to that of corn oil, suggesting that the A-2 might be the CLA-TG. The R_f value of the A-4 was similar to that of FCLA, indicating that A-4 might be FCLA. Based on these results, the A-2 and A-4 fractions would be the CLA-TG and FCLA, respectively, and the A-1 fraction is less polar than CLA-TG, whereas the A-3 and A-5 fractions are more polar than CLA-TG.

Five fractions (A-1, A-2, A-3, A-4, and A-5) were collected from the silica gel TLC plate and subjected to GC and spectral analysis for the identification.

Identification of CLA derivatives of glycerol

GC and UV analysis: GC analysis revealed that all fractions (A-1, 2, 3, 4 and 5) contained two major CLA isomers (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA). A typical GC chromatogram of A-2 is shown in Fig. 1 (top). The ratio of the CLA isomers in FCLA from five fractions was similar to that from the CLA sample, synthesized by the method of Kim et al. (19) (Fig. 1, bottom). All fractions exhibited a UV max at 233 nm in methanol, which is a typical UV absorbance of CLA. Consequently, these fractions are derivatives of CLA, except for A-4, which was identified as FCLA.

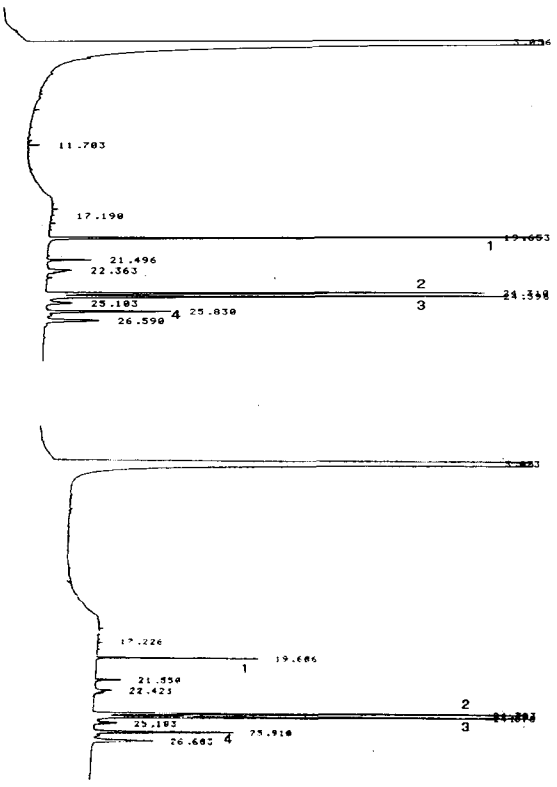

Fig. 1. GC chromatograms of fraction A-2 (top panel) and chemically-synthesized CLA from linoleic acid by alkaline isomerization (bottom panel). Peak identification : 1, $C_{17:0}$ (internal standard); 2, *cis*-9, *trans*-11 CLA; 3, *trans*-10,*cis*-12 CLA; 4, *trans*-9,*trans*-11 CLA and *trans*-10,*trans*-12 CLA.

Spectral analysis of A-1: The A-1 contained the signal of the conjugated diene protons (5.3 ~ 6.3 ppm). This fraction contained carbonyl (171 ~ 182 ppm), alkene (124 ~ 134 ppm) and alkane carbon (12 ~ 33 ppm) signals in ^{13}C-NMR. However, it did not have the signals of protons and carbons of glycerol molecule in ^{1}H-NMR spectrum. Thus, the A-1 is not a CLA derivative of glycerol. Molecular weight of the A-1 was 542 from mass spectral analysis, indicating A-1 is anhydrous form of CLA. Based on these spectral and TLC analysis data, the A-1 was identified as a CLA dimer.

Spectral analysis of A-2: In the IR spectrum, no stretch of -OH was seen, whereas the stretch of alkene and aliphatic alkane at 3000 ~ 2800 cm^{-1} and carbonyl stretch at 1750 cm^{-1} were observed. ^{1}H-NMR spectrum showed that this fraction contains signals of four conjugated diene protons of CLA at 5.3 ~ 6.3 ppm and also contained the signal of five protons of the glycerol backbone at around 4.1 ~ 4.4 ppm. The area ratio of the conjugated diene protons to the glycerol protons was a 12:5, indicating that three moles of CLA was esterified with one mole of glycerol. In ^{13}C-NMR, the A-2 contained carbonyl carbons (171 ~ 182 ppm), alkene carbon (124 ~ 134 ppm), alkane carbon (12 ~ 33 ppm) and glycerol carbon (60 ~ 68 ppm). Based on these data, the A-2 fraction was identified as a CLA-TG.

Spectral analysis of A-3: The A-3 possessed -OH (3357 cm^{-1}), alkane and alkene (3000~2800 cm^{-1}), and carbonyl (1750 cm^{-1}) groups in IR spectrum, indicating that OH group of glycerol molecule was not completely esterified with CLA. ^{1}H-NMR spectrum showed approximately a 8:6 ratio of conjugated diene protons to glycerol protons, revealing that 2 moles of CLA were esterified with one mole of glycerol. The molecular weight of the A-3 appeared to be 616 from mass spectral analysis, which is an equivalent molecular weight of two moles of CLA esterified with a glycerol. These spectral analysis data implied that the A-3 is a CLA-DG.

Spectral analysis of A-5: The A-5 possesses OH (3357 cm^{-1}), alkene and alkane (3000~2800 cm^{-1}), and carbonyl (1750 cm^{-1}) groups in the IR spectrum, indicating that glycerol OH was not completely esterified with CLA. The ratio of conjugated diene protons to glycerol protons was a 4:7, indicating that one mole of CLA was esterified with a glycerol molecule. It's molecular weight was found to be 354 from mass spectral analysis. Thus, the A-5 was identified as a CLA-MG.

An optimum reaction condition for the synthesis of CLA-TG

Table 1 shows the effect of solvents on the esterification of CLA with glycerol by Lipozyme. FCLA (500 mg) was reacted with glycerol (54.8 mg) in a 3 ml of solvents (pentane, hexane, and isooctane), containing Lipozyme (90 mg) by incubation in a shaking incubator (50°C) for 72 hr. Excess amount of Lipozyme was used to avoid the limitation of enzyme concentration. The yield of CLA-TG synthesized in pentane, hexane and isooctane was 18.1, 18.7 and 20.6%, respectively.

The yield of the CLA-TG was affected by the mole ratio of glycerol to CLA (500 mg). When the mole ratio (glycerol/CLA) increased up to a 20 in isooctane (3 ml), the yield of CLA-TG was increased, but not proportional to the ratio (Table 2). The resultant contained 51.6% CLA-TG at the mole ratio 7, but the CLA-TG content was not significantly elevated with the increase of the mole ratio of glycerol to CLA above 7. Such data indicate that the optimum mole ratio of glycerol to CLA in 3 ml isooctane is 7.

Table 1. Effect of solvents on the synthesis of CLA-derivatives of glycerol by Lipozyme[1)]

Solvent	CLA-TG		Others[4)]
	Amount (mg)[2)]	Yield (%)[3)]	
Pentane	90.5±2.2[5)]	18.1	409.5±4.3
Hexane	93.4±0.6	18.7	406.6±3.9
Isooctane	103.1±1.0	20.6	396.9±1.3

[1)]CLA, 500 mg; glycerol, 54.8 mg; solvent, 3 ml; Lipozyme, 90 mg; reaction for 72 hr at 50°C.
[2)]Amount (mg) of CLA contained in CLA-TG.
[3)]Percentage of CLA contained in TG form against total CLA (500 mg) used.
[4)]Amount (mg) of CLA contained in other form such as FCLA, CLA-DG, CLA-MG, and CLA dimer.
[5)]Mean±SD of three experimental data.

Table 2. Effect of the mole ratio of glycerol to CLA on the synthesis of CLA-TG by Lipozyme[1)]

Glycerol/CLA mole ratio	CLA-TG		Others[4)]
	CLA-TG (mg)[2)]	Yield (%)[3)]	
1/3[5)]	91.3±0.9[6)]	18.3	408.7±0.8
1	176.3±6.3	34.5	327.7±13.3
5	245.5±4.6	49.1	254.5±0.6
7	258.3±5.3	51.6	241.7±3.6
10	257.4±0.6	51.5	242.6±2.6
20	264.4±16.5	52.9	235.6±7.5

[1)]CLA, 500 mg; isooctane, 3 ml; Lipozyme, 90 mg; reaction for 72 hr at 50°C.
[2)]Amount (mg) of CLA contained in CLA-TG.
[3)]Percentage of CLA contained in TG form against total CLA (500 mg) used.
[4)]Amount (mg) of CLA contained in other form such as FCLA, CLA-DG, CLA-MG, and CLA dimer.
[5)]CLA 500 mg/glycerol (54.8 mg).
[6)]Mean±SD of three experimental data.

Table 3 shows that the effect of the amount of isooctane on the CLA-TG synthesis by Lipozyme (90 mg). With a 7 mole ratio of glycerol to CLA (1150 mg : 500 mg), CLA-TG amount synthesized was elevated by increasing the amount of isooctane up to 7 ml, but was not greatly elevated in more than 3 ml. Hence, the optimum amount of the isooctane was found to be a 3 ml under the test condition. Using the 7 mole ratio of glycerol to CLA dissolved in 3 ml isooctane, the effect of the amount of Lipozyme was examined for CLA-TG synthesis (Table 4). The amounts of Lipozyme affected the synthesis of CLA-TG at given conditions; 10 mg Lipozyme gave 41.8% CLA-TG and 150 mg Lipozyme gave 52.7% CLA-TG. However, the amount of CLA-TG synthesized by Lipozyme (25~150 mg) was ranging from 50.8% to 52.7%, indicating that at given condition the amount of Lipozyme more than 25 mg does not greatly increased the amount of CLA-TG synthesized.

Table 3. Effect of the amount of isooctane on the synthesis of CLA-TG by Lipozyme[1)]

Isooctane (ml)	CLA-TG		Others[4)]
	Amount (mg)[2)]	Yield (%)[3)]	
0	131.4±2.6[5)]	26.3	368.5±13.6
0.5	168.8±1.3	33.8	331.2±6.6
1	184.1±1.5	36.8	315.8±1.5
2	231.9±4.5	46.4	268.1±0.8
3	257.7±1.5	51.5	242.3±0.7
5	259.8±0.4	51.9	240.7±0.8
7	261.5±0.3	52.3	238.5±0.5

[1)]CLA, 500 mg; glycerol, 1150 mg; Lipozyme, 90 mg; reaction for 72 hr at 50°C.
[2)]Amount (mg) of CLA contained in CLA-TG.
[3)]Percentage of CLA contained in TG form against total CLA (500 mg) used.
[4)]Amount (mg) of CLA contained in other form such as FCLA, CLA-DG, CLA-MG, and CLA dimer.
[5)]Mean±SD of three experimental data.

Table 4. Effect of the amount of Lipozyme on the synthesis of CLA-TG[1)]

Lipozyme (mg)	CLA-TG		Others[4)]
	Amount (mg)[2)]	Yield (%)[3)]	
10	208.8±0.1[5)]	41.8	291.3±0.8
15	222.6±0.5	44.5	277.4±0.8
20	231.4±0.7	46.3	268.6±0.7
25	254.0±0.6	50.8	246.0±1.2
50	255.6±0.7	51.1	244.4±1.2
100	256.8±1.3	51.4	243.2±0.4
150	263.7±0.7	52.7	236.3±0.4

[1)]CLA, 500 mg; glycerol, 1150 mg; isooctane, 3 ml; reaction for 72 hr at 50°C.
[2)]Amount (mg) of CLA contained in CLA-TG.
[3)]Percentage of CLA contained in TG form against total CLA (500 mg) used.
[4)]Amount (mg) of CLA contained in other form such as FCLA, CLA-DG, CLA-MG, and CLA dimer.
[5)]Mean±SD of three experimental data.

To determine optimum reaction time, the reaction was carried out at the 7 mole ratio of glycerol to CLA (1150 mg: 500 mg) in isooctane (3 ml) by Lipozyme (50 mg) for a period of 72 hr (Table 5). Maximum CLA-TG (51.4%) was obtained from the reaction for 72 hrs, but the amount of the CLA-TG synthesized for 72 hrs was not greatly different from that produced from a 48 hr reaction.

Yield of CLA-TG at an optimum reaction condition

CLA-TG was synthesized in isooctane under the optimum reaction condition by Lipozyme-catalyzed esterification of CLA with glycerol (Table 6). The reaction mixture of glycerol (1150 mg), CLA (500 mg), isooctane (3 ml) and 50 mg Lipozyme was reacted for 48 hr in a shaking incubator (50°C and 200 rpm). The resultant was fractionated into CLA-TG, CLA-DG, CLA-MG, FCLA and CLA dimer by a precoated silica gel TLC followed by GC for quantitative analysis. The resultant

Table 5. Effect of reaction time on the synthesis of CLA-TG by Lipozyme[1)]

Time (hr)	CLA-TG		Others[4)]
	Amount (mg)[2)]	Yield (%)[3)]	
2	2.1±0.2[5)]	0.4	498.6±2.9
4	14.4±0.2	2.9	485.6±3.2
6	33.7±0.4	6.7	466.3±4.6
12	97.7±0.3	19.5	402.3±1.4
24	225.9±1.7	45.2	274.1±1.2
36	252.4±0.5	50.5	247.6±2.4
48	255.7±0.5	51.1	244.3±0.5
72	256.9±0.7	51.4	243.1±1.3

[1)]CLA, 500 mg; glycerol, 1150 mg; isooctane, 3 ml; Lipozyme, 50 mg; reaction at 50°C.
[2)]Amount (mg) of CLA contained in CLA-TG.
[3)]Percentage of CLA contained in TG form against total CLA (500 mg) used.
[4)]Amount (mg) of CLA contained in other form such as FCLA, CLA-DG, CLA-MG, and CLA dimer.
[5)]Mean±SD of three experimental data.

Table 6. Enzymatic synthesis of CLA-glycerol derivatives at a given optimal condition[1)]

CLA-glycerol derivatives	Amount (mg)	Yield (%)[2)]
CLA-TG	262.0±0.3[3)]	52.4
CLA-DG	155.4±0.4	31.1
CLA-MG	37.9±0.3	7.6
FCLA+CLA dimer	44.7±0.3	9.0

[1)]CLA, 500 mg; glycerol, 1150 mg; isooctane, 3 ml; Lipozyme, 50 mg; reaction for 48 hr at 50°C.
[2)]Percentage of CLA in CLA-glycerol derivatives against total CLA (500 mg) used.
[3)]Mean±SD of three experimental data.

contained 52.4% CLA-TG, 31.1% CLA-DG, 7.6% CLA-MG, and 9.0% FCLA plus CLA dimer. Amounts of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA were calculated as the percentage of CLA derivatives synthesized.

With few exceptions, most food matrices, including oils, dairy and animal, and other agricultural products, contain TG forms of fatty acids, not free fatty acids. To apply FCLA in such food systems, the FCLA synthesized from linoleic acid by microorganisms or chemicals should be converted to CLA-TG form. In the present study, the FCLA was converted to CLA-TG by Lipozyme. This enzyme has been used in industries for the esterification of long chain fatty acids to glycerol or certain alcohol and is commercially available at a low cost (20).

The esterification of CLA with glycerol by Lipozyme was affected by several factors: kinds and amount of solvent, the mole ratio of glycerol to CLA, the amount of Lipozyme and reaction time. The optimum condition for the synthesis of the CLA-TG is to react CLA (500 mg) with glycerol (1150 mg) in isooctane (3 ml) for 48 hr by Lipozyme (50 mg). For the efficiency of the synthesis, the mole ratio of glycerol to CLA is an important factor. It could be assumed that at least a 2.5 OH group in glycerol is necessary for the esterification of a -COOH group in CLA under the reaction condition of the 7 mole ratio. The reaction temperature is also an important factor. The reaction temperature was 50°C rather than 60°C, which was provided by the manufacturer for the protection of CLA from oxidation, due to the oxidative stability of CLA. Under the optimum esterification condition, approximately 52% CLA was esterified with glycerol.

Synthetic CLA contained at least 8 CLA isomers; approximately 46% *cis*-9,*trans*-11 CLA and 48% *trans*-10,*cis*-12 CLA isomers as major isomers (Fig. 1, bottom). The CLA-TG contained the two major CLA isomers without positional distinguishment on the OH group of glycerol for the isomers. Recently, it was reported that the *trans*-10,*cis*-12 CLA isomer was more effective in the reduction of body fats than the *cis*-9,*trans*-11 CLA suggesting that each isomer possesses different biological activities (21). Therefore, further research is necessary to the synthesis of CLA-TG containing either the *trans*-10,*cis*-12 CLA or *cis*-9,*trans*-11 CLA.

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