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A Simple Method for the Preparation of Highly Pure Conjugated Linoleic Acid (CLA) Synthesized from Safflower Seed Oil

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Abstract

A simple and rapid method was developed to prepare a large quantity of highly pure conjugated linoleic acid (CLA) chemically-synthesized from safflower seed oil (SSO). CLA-SSO (74.9% in purity) was synthesized from fresh SSO (79.9% of linoleic acid) by alkaline isomerization at 180°C. Urea (50 g) and CLA-SSO (25 g) were completely dissolved in ethanol (750 ml) using a water bath (50°C) and followed by refluxing for 60 min. The resultant was cooled to room temperature and stored in a cold room (4°C) for 24 hrs. After removing the urea adduct by filtration, the filtrate was rotoevaporated under 40°C and the residue was dissolved in hexane (200 ml). The hexane extract was washed with distilled water (100 ml × 3) and dried over sodium sulfate anhydrous. This urea treatment procedure was repeated three times. The purity of CLA recovered from the hexane extract was 95.0%. This method can be applied to prepare a large quantity of highly pure chemically-synthesized CLA (>0.5 kg/a batch) from any plant oils containing high percentages (>70%) of linoleic acid.

Key words: conjugated linoleic acid (CLA), urea adduct

INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for a group of positional (C8,C10; C9,C11; C10,C12, and C11,C13) and geometric (*cis, cis; cis, trans; trans, cis*; and *trans, trans*) isomers of linoleic acid with a conjugated double bond system (1,2). It was first identified as comprising an anticarcinogenic principal present in grilled ground beef (1). Later, it was found in many other food sources, especially dairy products (3-6).

Chemically-synthesized CLA from linoleic acid, composed of approximately 48% each of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA, inhibited carcinogen-induced neoplasia in several animal models (1,7-10). Other significant biological activities, such as the immune stimulation (11,12), body fat reduction (13,14) and modulation of cholesterol content in bood (15) were evident.

Currently, scientists are using CLA as medicinal ingredients, and as food and feed additives (16,17). Thus, the demand for CLA is steadily increasing in Korea and other countries. Although little amount of *cis*-9,*trans*-11 CLA can be synthesized from linoleic acid by certain bacterial species (18), a large quantity of CLA, consisting of approximately 47% *cis*-9,*trans*-11 CLA and 48% *trans*-10,*cis*-12 CLA, is synthesized from linoleic acid by alkaline isomerization at high temperature (19,20). Reagent grade of CLA (95% *cis*-9,*trans*-11 CLA plus *trans*-10,*cis*-12 CLA) is commercially available at $100/g (21). Feed-grade CLA, relatively low in purity, is synthesized from soybean or corn oil (about 50% linoleic acid) without further purification (16,17).

Food grade CLA (>90% in purity), commercially not available, might be synthesized from certain plant seed oils, containing high percentages of linoleic acid (>70%), such as safflower seed oil (SSO) and sunflower seed oil. SSO could be a better candidate for a source of the CLA due to a high percentage of linoleic acid (about 80%) and abundancy in Korea. The CLA synthesized from SSO (CLA-SSO) by alkaline isomerization contains about 80% CLA and 20% other substances, including saturated fatty acids and impurities produced by the isomerization process (20,22). Hence, the purification step is necessary to increase the purity of the CLA as food additives.

Urea is a very useful material to concentrate certain polyunsaturated fatty acids by removing saturated fatty acids present in the fatty acid pool (20,23,24). Generally, fatty acid methylesters are applied to the concentration procedure of unsaturated fatty acids from a fatty acid pool by urea adducts. Unlike normal unsaturated fatty acids, CLA isomers, especially *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA, are readily isomerized to their corresponding *trans, trans* isomers and decomposed to artifacts by methylesterification processes. Therefore, the free form of CLA must be applied to the purification process to maintain the correct composition of CLA isomers.

In the present study, a fast and inexpensive purification method was developed to obtain a large quantity of highly pure CLA. The free form of CLA was synthesized from SSO by alkaline isomerization at 180°C, and was further purified

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by urea. The present method could be applied to purify 1 kg of CLA-SSO to get more than 0.5 kg CLA per batch with a purity greater than 95%.

MATERIALS AND METHODS

Materials

The SSO was prepared by pressing the fresh safflower seeds. CLA was chemically-synthesized from SSO by alkaline isomerization at 180°C according to the method described by Kim et al. (20). Urea, KOH, and ethylene glycol were obtained from Shinyo Co. (Osaka, Japan). Ethanol, methanol and hexane were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All other chemicals used were reagent grade.

Urea treatment of CLA-SSO

Urea (50 g) was added to the CLA-SS0 (25 g) dissolved in ethanol (500, 750, or 1000 ml), followed by refluxing for 60 min. The resultant was stored at 4°C for 24 hrs to produce urea adducts which were removed by filtration. The filtrate was rotoevaporated and the residue was redissolved in hexane (200 ml), followed by washing with distilled water (100 ml × 3). The hexane extract was rotoevaporated after removing the residual water by sodium sulfate anhydrous. These urea treatment procedures were repeated three times.

Preparation of CLA methylester (CLA-Me)

H_2SO_4-catalyzed methylesterification

0.05 N H_2SO_4/ methanol solution (3 ml) was added to a screw-cap test tube (15 ml) containing the CLA (<50 mg) and heptadecanoic acid (1 mg) as an internal standard (IS) and the solution in a test tube was agitated with a vortex mixer for 30 sec. The test tubes, capped tightly with a teflon-lined screw cap under nitrogen and wrapped with aluminum foil to prevent light exposure, were heated in a boiling water bath for various reaction times (5, 10, 20, and 50 min). After cooling the test tube to room temperature, CLA-Me was extracted with hexane (3 ml × 3) by vigorous hand-shaking for one min. The hexane extract, washed with distilled water (3 ml × 3), was dried over Na_2SO_4 anhydrous (approximately 200 mg), and then used as the sample for the analysis of CLA by the GC described below.

Lipozyme-catalyzed methylesterification

Lipozyme (10% H_2O) and hexane (2 ml) were added to the screw-cap test tube containing free CLA (50 mg) in methanol (0.6 ml). The sample test tube, screw-capped and filled with nitrogen gas, was incubated in a shaking incubator (60°C, 170 rpm) for 2 hrs. After completing the reaction, CLA-Me was extracted and purified according to the procedure described above for the H_2SO_4-catalyzed methylesterification method.

GC analysis

CLA-Me was analyzed by a Hewlett Packard 5890 GC equipped with a FID, a Supelcowax 10 fused capillary column (60 m × 0.32 mm, i.d., 25 μm film thickness) and a Hewlett Packard 5890 integrator. Oven temperature was increased from 50 to 200°C at a rate of 10°C/min. The injector port and detector temperatures were set at 260°C. N_2 was used as a carrier gas at a flow rate of 2 ml/min.

RESULTS AND DISCUSSION

The SSO is one of the abundant plant-seed oils, containing a high percentage of linoleic acid (>75%). Linoleic acid present in SSO is easily converted to CLA by treatment of KOH at 180°C (20,22). Fig. 1 represents GC chromatograms of SSO methylester (SSO-Me, Fig. 1A), and CLA-SSO methylester (CLA-SSO-Me, Fig. 1B) synthesized by the alkaline isomerization method (22), and methyl ester of urea-treated CLA-SSO (CLA-SSO-Me, Fig. 1C). Fatty acid profiles of the SSO and the CLA-SSO are shown in Table 1. SSO contained 79.9% linoleic acid, 12.2% oleic acid, 2.7% stearic acid, and 5.2% palmitic acid as major fatty acids. Almost all of the linoleic acid in the SSO was converted to CLA by the isomerization, while other fatty acids were not affected. Of the CLA-SSO isomers (80.2%), *cis*-9,*trans*-11 CLA (33.4%) and *trans*-10,*cis*-12 CLA (41.5%) were predominant, but other CLA isomers (5.3%), including *cis, cis* CLA and *trans, trans* CLA, were minor.

Highly pure CLA, a mixture of approximately 47% *cis*-9, *trans*-11 CLA and 48% *trans*-10,*cis*-12 CLA isomers, can usually be obtained from the alkaline isomerization of pure linoleic acid (21). However, such a pure CLA might be pre-

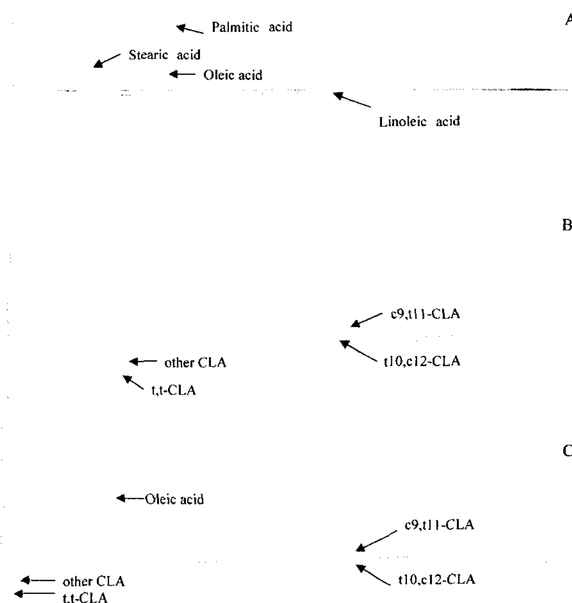

Fig. 1. GC chromatograms of fresh SSO-Me (A) and crude CLA-SSO-Me (B), and CLA-SSO-Me treated with 50 g urea dissolved in 750 ml ethanol (C). Other CLA means largely *cis,cis* CLA isomers. Identification of CLA isomers was based on the data shown in the report of Ha et al. (3).

Table 1. Composition of CLA isomers of SSO, CLA-SSO and urea-treated CLA-SSO[1)]

Major fatty acid	SSO	CLA-SSO[2)]	Urea-treated CLA-SSO in ethanol (ml)[3)]		
			500	750	1,000
C16:0	5.2±0.3[4)]	5.1±0.2	tr[5)]	tr	0.7±0.2
C18:0	2.7±0.5	1.7±0.4	tr	tr	tr
C18:1	12.2±0.8[a6)]	11.8±0.5[a]	7.2±1.1[a]	3.6±0.1[c]	6.4±0.9[b]
C18:2	79.9±1.2	tr	tr	tr	tr
cis-9,*trans*-11 CLA	tr	33.4±0.8[a]	35.8±0.7[b]	38.8±0.2[c]	36.1±0.6[b]
trans-10,*cis*-12 CLA	tr	41.5±1.0[a]	53.3±0.6[b]	56.2±0.1[c]	52.3±1.0[b]
Other CLA[7)]	tr	2.4±0.2	tr	tr	1.5±0.8
trans,trans-CLA	tr	2.9±0.1	2.2±0.5	tr	1.2±0.4
AMC[8)]	tr	1.2±0.2	1.5±0.3	1.4±0.1	1.5±0.2

[1)]Sample was methyl-derivatized by H_2SO_4-catalyzed methylesterification described in the Method. Composition (area percentage) of a given fatty acid was calculated based on the ratio of the area of the given fatty acid to the total area of the fatty acids calculated.
[2)]CLA-SSO was synthesized from SSO by the alkaline isomerization method.
[3)]Free form of CLA-SSO (25 g) dissolved in a given amount (ml) of ethanol was treated with urea (50 g) as described in the Method.
[4)]Mean±SD of three experimental data.
[5)]tr means trace: area counts are less than 500 by Hewlett Packard detector.
[6)]Different superscript letters in the same row mean significantly different at $p<0.05$ by t-test.
[7)]Other CLA represents mainly *cis,cis* CLA isomer.
[8)]AMC stands for allylmethoxy CLA produced during methylesterification as an artifact.

pared by removing impurities in the SSO-CLA. Urea can effectively remove saturated fatty acids present in the unsaturated fatty acid pool by urea adduct formation. For the purpose of the removal of the impurities (25.1%), including *cis,cis* and *trans,trans* CLA isomers, present in the CLA-SSO sample, the CLA-SSO (25 g) dissolved in ethanol (750 ml) was treated with urea (50 g) by refluxing for 60 min. The urea adducts formed at low temperature (4°C) were removed by filtration. Saturated fatty acids, such as palmitic and stearic acids, were completely removed from the CLA-SSO sample as well as *cis,cis* and *trans,trans*-CLA isomers. The substantial amount of oleic acid (Fig. 1C, Table 1) was also removed resulting in the elevation of the purity of CLA-SSO (*cis*-9,*trans*-11 CLA plus *trans*-10,*cis*-12 CLA) from 74.9% to 95.0% (Table 1).

Urea adduct formation with saturated fatty acids was highly dependant upon the concentration of urea in ethanol and the amount of saturated fatty acids present in the sample. Most of the impurities (5.03 g) present in the CLA-SSO (25 g) were successfully removed by urea (50 g) dissolved in ethanol (750 ml) (Table 1, Fig. 1C), whereas the impurities were not successfully removed by increasing and decreasing the ethanol volume from 750 ml to 500 ml and 1000 ml, respectively (Table 1). The purity of CLA-SSO (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) treated with 50 g urea in 750 ml ethanol was 95.0%, whereas the purity of CLA-SSO treated with the urea dissolved in 500 ml and 1000 ml ethanol were 89.1 and 88.4%, respectively. The ethanolic urea solution composed of 50 g urea and 750 ml ethanol successfully removed all saturated fatty acids and *cis, cis* CLA and *trans, trans* CLA which are similar to stearic acid in chemical structure from CLA-SSO, but other treatments were not. These results indicate that the ethanolic urea solution composed of 50 g urea and 750 ml ethanol is suitable for the purification of 25 g CLA-SSO.

Most importantly, the present method utilized the free form of CLA-SSO for the purification process, while most other methods for the concentration of certain unsaturated fatty acids utilized methylester forms (23,24). Unlike normal unsaturated fatty acids, CLA, the *cis*-9,*trans*-11 and *trans*-10, *cis*-12 CLA isomers easily converted to their corresponding *trans, trans* CLA isomers and allylmethoxy CLA (AMC) during the preparation of methylesterification, without extensive care. Hence, it is impossible to get an accurate composition of CLA isomers if CLA-Me is used for the urea-adduct purification method. In addition, the methylesterification process is time consuming.

Table 2 shows the composition of the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers of the CLA-SSO, which was purified as the free CLA-SSO form and the CLA-SSO-Me form, methylesterified with H_2SO_4, by the ethanolic urea solution (50 g urea + 750 ml ethanol). The true composition of CLA- SSO isomers was determined from the CLA-SSO-Me prepared with Lipozyme. No significant difference in the purity (especially, *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) of the CLA-SSO, purified as free and methylester forms of CLA-SSO, was observed, indicating that the forms of CLA do not affect the purity under the purification condition. However, the composition of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers was greatly affected by the purification of the CLA-SSO-Me prepared with the H_2SO_4-catalyzed esterification, especially decreased in the composition of the *cis*-9,*trans*-11 CLA isomer as compared to that with Lipozyme. The compositional change of CLA-SSO occurred during the H_2SO_4-catalyzed methylesterification process, not by the urea-adduct purification process. Thus, the present method, utilizing the free form of CLA, provides a faster and more accurate composition of CLA isomers than the method utilizing the

Table 2. Effects of CLA-SSO forms on the purity of the CLA-SSO purified by the urea-adduct method[1)]

Major fatty acid	CLA-SSO-Me by lipozyme[2)]	Form of CLA-SSO treated with urea	
		Free[3)]	Methylester[4)]
C16:0	6.3±0.8[5)]	tr[6)]	tr
C18:0	1.9±0.1	tr	tr
C18:1	9.5±1.0[a7)]	3.6±0.1[b]	2.7±0.3[b]
C18:2	0	0	0
cis-9,*trans*-11 CLA	43.4±0.8[a]	51.3±0.2[b]	37.1±0.2[c]
trans-10,*cis*-12 CLA	38.5±0.3[a]	43.7±0.1[b]	58.8±0.1[c]
Other CLA[8)]	0.4±0.1	tr	tr
trans,trans-CLA	tr	tr	tr
AMC[9)]	tr	1.4±0.1	1.4±0.1

[1)]Composition of the given fatty acid was calculated based on the ratio of the area of the given fatty acid to the total area of the fatty acids calculated.
[2)]CLA-SSO-Me was prepared with Lipozyme described in the Method.
[3)]Free form of CLA-SSO was treated with urea and then, methylesterified with Lipozyme to analyze the composition of fatty acids by GC.
[4)]CLA-SSO-Me prepared by 0.05 N H_2SO_4-catalyzed methylesterification was directly treated with urea and then, the composition of fatty acids was analyzed by GC.
[5)]Mean±SD of three experimental data.
[6)]The tr means less than 500 area counts by the Hewlett Packard recorder.
[7)]Different superscript letters in the same row mean significantly different at $p<0.05$ by t-test.
[8)]Other CLA represents mainly *cis,cis* CLA isomer.
[9)]AMC stands for allylmethoxy CLA produced during methylesterification as an artifact.

methylester form.

CONCLUSION

The purity (74.9% *cis*-9,*trans*-11 CLA plus *trans*-10,*cis*-12 CLA) of the CLA-SSO (25 g) treated with urea (50 g) dissolved in ethanol (750 ml) increased to 95%. This purification method using the free form of CLA is fast and inexpensive, and provides an accurate composition of CLA isomers. This method is applicable to purify the CLA synthesized from plant oils, containing a high percentage of linoleic acid, and produces more than 0.5 kg CLA (95.0% in purity) per batch, using a lab-scale facility.

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