# Enrichment of CLA Isomers by Selective Esterification with L-Menthol Using *Candida rugosa* Lipase

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**ABSTRACT:** Commercially available preparations of CLA are composed of almost equal amounts of 9-*cis*,11-*trans* (9*c*,11*t*)-CLA and 10-*trans*,12-*cis* (10*t*,12*c*)-CLA. Each isomer was fractionated and enriched, for availability as a food supplement, by a process comprising selective esterification with L-menthol by *Candida rugosa* lipase, distillation, and *n*-hexane extraction. The first selective esterification of CLA isomers was conducted with an equimolar amount of L-menthol at 30°C. The oil phase of the reaction mixture was fractionated into an L-menthyl ester fraction (9*c*,11*t*-CLA rich) and an FFA fraction (10*t*,12*c*-CLA rich) by distillation. The FFA fraction was esterified again with an equimolar amount of L-menthol to enrich 10*t*,12*c*-CLA. The 10*t*,12*c*-CLA preparation was obtained as the resulting FFA fraction by distillation. 10*t*,12*c*-CLA was enriched to 91% with 40% recovery. To enrich 9*c*,11*t*-CLA, the L-menthyl ester fraction in the first esterification was chemically hydrolyzed, and the resulting FFA were esterified again with an equimolar amount of L-menthol. The 9*c*,11*t*-CLA preparation was obtained by chemical hydrolysis of the resulting L-menthyl ester fraction, followed by *n*-hexane extraction. 9*c*,11*t*-CLA was enriched to 94% with 42% recovery. This effective process for purification of CLA isomers using L-menthol is applicable to the production of food supplements.

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**KEY WORDS:** *Candida rugosa* lipase, CLA, L-menthol, purification, selective esterification.

CLA is a group of $C_{18}$ FA containing a pair of conjugated double bonds. A commercially available CLA preparation has been manufactured by alkali conjugation of safflower oil in propylene glycol, and the CLA mixture (referred to as FFA-CLA) is composed of almost equal amounts of 9-*cis*,11-*trans* (9*c*,11*t*)-CLA and 10-*trans*,12-*cis* (10*t*,12*c*)-CLA. The FFA-CLA has various physiological activities, such as the reduction of cancer incidence (1–3), beneficial effects on atherosclerosis (4,5), a decrease in body fat content (6,7), and improvement of immune functioning (8). Recently, it was reported that each isomer has different physiological activities: 9*c*,11*t*-CLA has anticancer activity (9); 10*t*,12*c*-CLA has activities to decrease body fat content (10–12), to increase energy expenditure (13), and to suppress the development of hypertension (14). To develop nutraceuticals containing the two CLA isomers at arbitrary contents, a large-scale fractionation process of the CLA isomers for availability as food supplements has been strongly desired.

Haas *et al*. (15) and McNeill *et al*. (16) fractionated two CLA isomers by selective esterification or hydrolysis using *Geotrichum candidum* lipase, which recognized 9*c*,11*t*-CLA more readily than 10*t*,12*c*-CLA. However, the processes included some drawbacks, such as low recovery of each isomer (15) and low purity (16). In addition, the lipase could not be applied for food production. *Candida rugosa* lipase, which is relatively inexpensive and available as a catalyst for food production, also acts on 9*c*,11*t*-CLA more strongly than on 10*t*,12*c*-CLA, as does *G. candidum* lipase. Esterification of FFA-CLA with lauryl alcohol using this lipase is also very effective to fractionate the two isomers, and they were highly fractionated by a process comprising selective esterification, distillation, and urea adduct fractionation (17,18). However, lauryl alcohol and urea cannot be used for the production of food supplements.

Selective hydrolysis of acylglycerols containing the two CLA isomers using *C. rugosa* lipase to fractionate the two isomers was recently reported (19). This process can be applied in the production of the two CLA isomers as food supplements, but their purities are not high. We thus aimed to develop an efficient process for the production of purified CLA isomers available as food supplements. L-Menthol is available for food production and can be used instead of lauryl alcohol. We recently showed that the *C. rugosa* lipase also catalyzes the esterification of FFA with L-menthol more efficiently than do other lipases (20,21). Furthermore, the M.W. of L-menthol, CLA, and CLA L-menthyl esters are 156, 280, and 418, respectively. The differences between them are larger than 100, and their separation is expected to be achieved by distillation. We thus paid attention to the selective esterification of FFA-CLA with L-menthol. This paper shows that the esterification of FFA-CLA with L-menthol by *C. rugosa* lipase is effective for fractionating the two CLA isomers.

## MATERIALS AND METHODS

*Materials.* FFA-CLA was a commercial product (CLA-80; Nisshin OilliO Group, Ltd., Tokyo, Japan) obtained by alkali

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conjugation of safflower oil in propylene glycol. Contents of the product were 33.3 wt% 9*c*,11*t*-CLA, 34.2 wt% 10*t*,12*c*-CLA, 1.1 wt% 9*c*,11*c*-CLA, 1.1 wt% 10*c*,12*c*-CLA, 2.7 wt% other CLA isomers, 16.3 wt% oleic acid, 6.4 wt% palmitic acid, 2.7 wt% stearic acid, and 2.2 wt% other FA. L-Menthol was purchased from Tokyo Chemical Industries (Tokyo, Japan). *Candida rugosa* lipase (Lipase-OF) was a gift from Meito Sangyo Co. (Aichi, Japan) and was composed of only isozyme-3 (22). One unit (U) of the enzyme activity was defined as the amount of lipase that liberated 1 μmol FA per minute in the hydrolysis of olive oil.

*Reactions.* A small-scale reaction was conducted in a 50-mL vessel. To determine the reaction conditions, a 4-g mixture of FFA and L-menthol at various molar ratios, 1 mL water (20 wt%), and various amounts of *C. rugosa* lipase were incubated at 30°C for 16–70 h with stirring at 500 rpm under a nitrogen atmosphere. A large-scale reaction was performed at 30°C in a 2-L reactor (MDS-U; Marubishi Bioengineering Co. Ltd., Tokyo, Japan) for 40 h with stirring at 350 rpm under a nitrogen atmosphere. The degree of esterification of the total FA (referred to as total esterification) was determined by titrating the amount of unesterified FFA with 0.1 N KOH solution. The degree of esterification of each CLA isomer was determined from the total esterification and the content of the isomers in L-menthyl esters by GC as described later.

*Fractionation of FFA and L-menthyl esters in the reaction mixture.* A small-scale fractionation of FFA and L-menthyl esters was performed by *n*-hexane extraction. The oil phase of the reaction mixture (*ca*. 3 g) containing FFA, L-menthyl esters, and L-menthol was mixed with 50 mL of 0.5 N KOH (20% ethanol solution). To the mixture was added 100 mL *n*-hexane, and the L-menthyl esters and L-menthol were extracted twice with *n*-hexane. FFA were extracted with 100 mL *n*-hexane twice after acidification of the aqueous phase to pH 2 with 1.0 N HCl.

A large-scale fractionation of the oil phase was performed with a short-path distillation apparatus (Wiperene Type 2-03; Kobelco Eco-Solutions Co. Ltd., Hyogo, Japan). Prior to applying short-path distillation, L-menthol was removed from the oil phase by simple distillation at 150°C and 270 Pa. The distillation residue was separated into FFA and L-menthyl ester fractions by three-step short-path distillation: the first step at 160°C and 27 Pa; the second step at 175°C and 27 Pa; the third step at 190°C and 27 Pa. The three distillates were combined, and the mixture was used as the FFA fraction. The residue was used as the L-menthyl ester fraction.

*Recovery of FFA from the L-menthyl ester fraction.* FFA were recovered from L-menthyl esters by chemical hydrolysis, followed by *n*-hexane extraction. The hydrolysis was conducted as follows: L-Menthyl esters (50 g) were dissolved in 800 mL ethanol solution containing 22 mL water and 14 g NaOH. The mixture was heated at 50°C for 40 min with occasional stirring under a nitrogen atmosphere. After hydrolysis, 800 mL water was added, and L-menthol was extracted twice with 500 mL *n*-hexane. The aqueous phase after extraction was acidified to pH 2 with 1.0 N HCl. FFA were then extracted twice with 500 mL *n*-hexane, and the solvent was evaporated.

*GC analyses.* Prior to GC analysis, the mixture of FFA and L-menthyl esters was fractionated into the FFA and L-menthyl ester fractions by *n*-hexane extraction, and they were methylated separately. FFA were methylated in 3 mL methanol containing 2% HCl by heating at 75°C for 5 min. L-Menthyl esters underwent methanolysis by heating at 75°C in 3 mL methanol containing 1% sodium methoxide for 30 min. FA contents in the FFA and L-menthyl ester fractions were analyzed with an Agilent 6890N gas chromatograph (Palo Alto, CA) connected to a DB-23 column (0.25 mm × 30 m; J&W Scientific, Folsom, CA). The column temperature was raised from 160 to 220°C at 2°C/min. The injector and detector temperatures were 245 and 250°C, respectively. The contents of L-menthol, FFA, and L-menthyl esters in the oil phase of the reaction mixture were analyzed with a Shimadzu GC-18A (Kyoto, Japan) connected to a DB-1ht column (0.25 mm × 5 m, J&W Scientific). The column temperature was raised from 80 to 370°C at 15°C/min, and the injector and detector temperatures were 390 and 370°C, respectively.

## RESULTS AND DISCUSSION

*Fractionation of CLA isomers by selective esterification.* FFA-CLA was esterified at 30°C with L-menthol under various conditions: molar ratio of FFA-CLA/L-menthol, 3:1–1:5; amount of lipase, 25–3000 U/g-mixture; reaction period, 16–70 h. Figure 1 shows the relationship between the degree of esterification of each isomer and total esterification. *Candida rugosa* lipase acted strongly on 9*c*,11*t*-CLA and weakly on 10*t*,12*c*-CLA in the esterification of FFA-CLA with L-menthol. The relationships were not affected by reaction conditions such as the molar ratio of FFA-CLA/L-menthol, the amount of lipase, and the reaction period, and the fractionation efficiencies of 9*c*,11*t*- and 10*t*,12*c*-CLA were determined only by total esterification. The results indicated that *ca*. 40–50% total esterification most efficiently fractionated 9*c*,11*t*-CLA into the L-menthyl ester fraction and 10*t*,12*c*-CLA into the FFA fraction.

Purities of 10*t*,12*c*-CLA in the FFA fraction and 9*c*,11*t*-CLA in the L-menthyl ester fraction were plotted against total esterification (Fig. 2). In this paper, purity was defined as the content of each CLA isomer based on the total contents of the two isomers (wt%). The purity of 10*t*,12*c*-CLA in the FFA fraction increased with increasing total esterification; reached a maximum value (83%) at 40–50% total esterification; and decreased thereafter (Fig. 2A). The recovery of 10*t*,12*c*-CLA in the FFA fraction decreased a little at a low degree of total esterification and decreased significantly after total esterification exceeded 40% (recovery at 40% total esterification, 95%).

The purity of 9*c*,11*t*-CLA in the L-menthyl ester fraction decreased slightly below 40% total esterification (purity at 40% total esterification, 89%) and decreased above 40% total

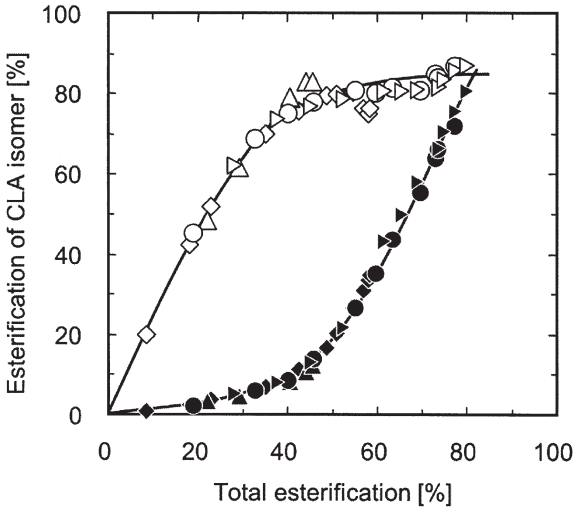



**FIG. 1.** Relationship between the degree of esterification of each isomer and total esterification in selective esterification under various conditions. A mixture of FFA-CLA/L-menthol (3:1–1:5, mol/mol), 20 wt% water, and 25–3000 U/g-mixture of *Candida rugosa* lipase was agitated at 30°C for 16–70 h. ◇, Reaction for 16 h with FFA-CLA/L-menthol (1:1, mol/mol) and various amounts of lipase; ○, reaction for 40 h with FFA-CLA/L-menthol (1:1, mol/mol) and various amounts of lipase; ▷, reaction for 70 h with various amounts of lipase; △, reaction for 24 h with various molar ratios of FFA/L-menthol (3:1–1:5, mol/mol) and 200 U/g-mixture lipase. The open and closed symbols show 9*c*,11*t*-CLA and 10*t*,12*c*-CLA, respectively.

esterification (Fig. 2B). Recovery of 9*c*,11*t*-CLA in the L-menthyl ester fraction corresponded to the degree of esterification of 9*c*,11*t*-CLA: The recovery reached 75% at 40% total esterification. From these results, it was most appropriate to cease the reaction at 40–50% total esterification to fractionate the two isomers efficiently and to achieve a good recovery of the two isomers. Hence, the conditions in the esterification were set as follows: L-menthol/FFA-CLA, 1:1 (mol/mol); water, 20 wt%; *C. rugosa* lipase, 200 U/g-mixture; temperature, 30°C; reaction period, 40 h.

These reaction conditions were applied to a small-scale fractionation of the two CLA isomers using 51.4 g FFA-CLA (total esterification after 40 h, 45%). The subsequent *n*-hexane extraction of the reaction mixture recovered 24.6 g FFA (recovery of 10*t*,12*c*-CLA, 81%) and 30.6 g L-menthyl ester/L-menthol (recovery of 9*c*,11*t*-CLA, 69%). The purity of 10*t*,12*c*-CLA in the FFA fraction was raised to 83%, and the purity of 9*c*,11*t*-CLA in the L-menthyl ester fraction was raised to 85%. To further increase the purities, FA in the two fractions were esterified again as described in the following sections.

*Enrichment of 10t,12c-CLA by esterification of the FFA fraction.* The FFA obtained by *n*-hexane extraction were mixed with an equimolar amount of L-menthol, 20 wt% water, and *ca*. 50–2000 U/g-mixture of lipase, and the mixture was stirred at 30°C for 40 h (Fig. 3). The purity of 10*t*,12*c*-CLA reached a maximum (91%) at 36% total esterification (recovery, 78%). The purity was sufficiently high compared with that of the selective hydrolysis of acylglycerols (purity, 80%) (19). Based on these results, we planned to stop the esterification at 35% total esterification. The reaction conditions were fixed as follows: L-menthol/FFA, 1:1 (mol/mol); water, 20 wt%; *C. rugosa* lipase, 200 U/g-mixture; temperature, 30°C; reaction time, 40 h. Under these conditions, 24.0 g FFA were esterified (total esterification, 33%). The subsequent *n*-hexane extraction recovered 15.2 g FFA (recovery of 10*t*,12*c*-CLA, 58%). These procedures increased the purity of 10*t*,12*c*-CLA to 91%.

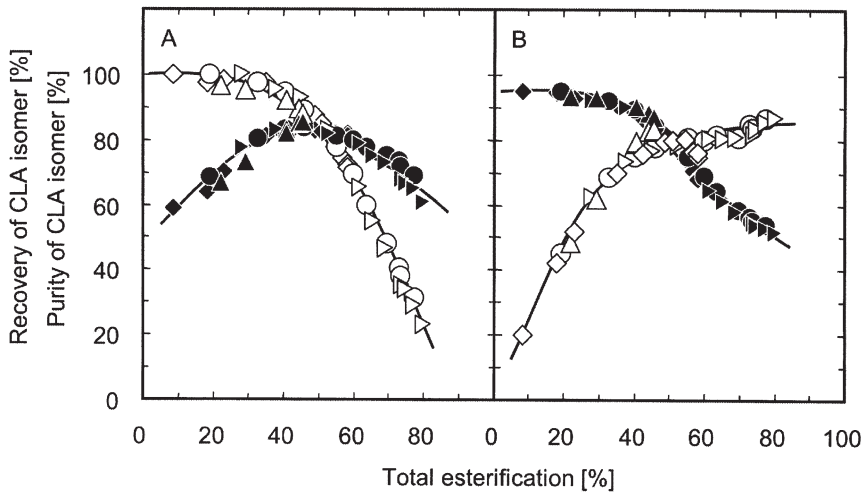



**FIG. 2.** Dependencies of the purity and recovery of (A) 10*t*,12*c*- and (B) 9*c*,11*t*-CLA on total esterification. A mixture of FFA-CLA/L-menthol (3:1–1:5, mol/mol), 20 wt% water, and 25–3000 U/g-mixture of *C. rugosa* lipase was agitated at 30°C for 16–70 h. ◇, Reaction for 16 h with FFA-CLA/L-menthol (1:1, mol/mol) and various amounts of lipase; ○, reaction for 40 h; ▷, reaction for 70 h; △, reaction for 24 h with various molar ratios of FFA/L-menthol (3:1–1:5, mol/mol) and 200 U/g-mixture lipase. The open and closed symbols show the recovery and purity of each isomer, respectively.

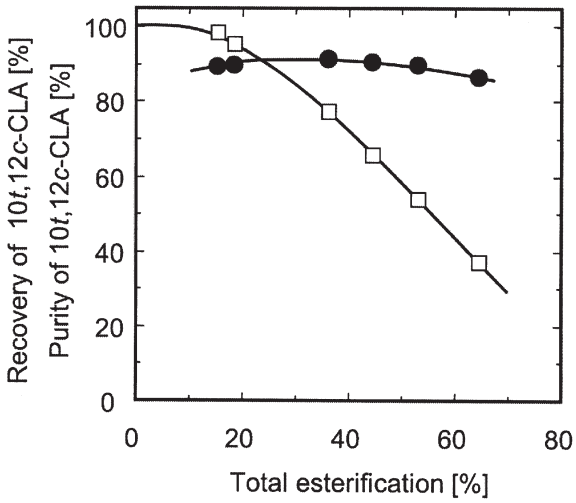



**FIG. 3.** Enrichment of 10*t*,12*c*-CLA in the FFA fraction by repeated selective esterification. A mixture of 2.58 g FFA obtained by *n*-hexane extraction, 1.42 g L-menthol, 1 mL water, and 50–2000 U/g-mixture of *C. rugosa* lipase was stirred for 40 h at 30°C. ●, Purity of 10*t*,12*c*-CLA; □, recovery of 10*t*,12*c*-CLA.

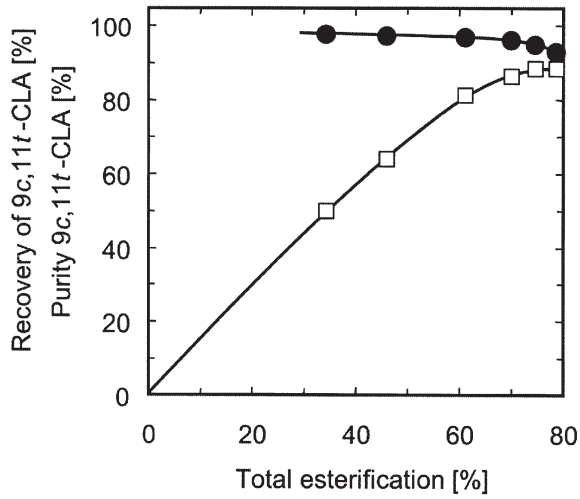
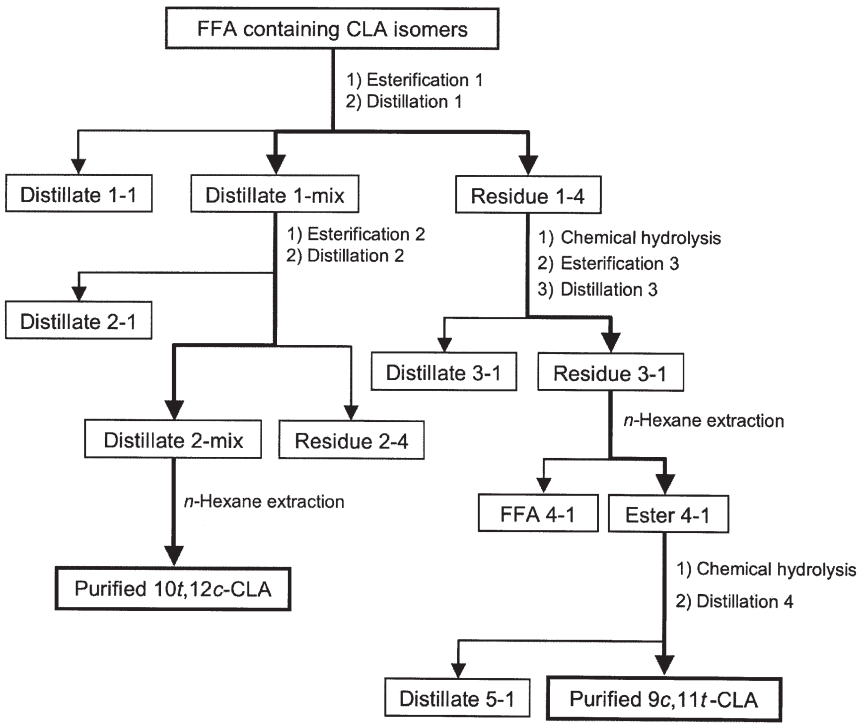



**FIG. 4.** Enrichment of 9*c*,11*t*-CLA in the ester fraction by repeated selective esterification. After chemical hydrolysis of L-menthyl ester obtained by *n*-hexane extraction, a mixture of 2.58 g resulting FFA, 1.42 g L-menthol, 1 mL water, and 25–1500 U/g-mixture of *C. rugosa* lipase was stirred for 40 h at 30°C. ●, Purity of 9*c*,11*t*-CLA; □, recovery of 9*c*,11*t*-CLA.

*Enrichment of 9c,11t-CLA by esterification of FA in the L-menthyl ester fraction.* We attempted to hydrolyze the L-menthyl ester through *C. rugosa* lipase-catalyzed selective hydrolysis, but an efficient hydrolysis could not be achieved (hydrolysis, <10%). This phenomenon may have been due to the facts that the reaction system was biphasic and the equilibrium position apparently leaned toward esterification by several factors. Hence, L-menthyl esters were chemically hydrolyzed with NaOH in the presence of ethanol, and the resulting FFA were esterified again. The esterification was performed at 30°C by stirring a mixture of FFA/L-menthol (1:1, mol/mol), 20 wt% water, and 25–1500 U/g-mixture of lipase for 40 h. The purity of 9*c*,11*t*-CLA in the L-menthyl ester fraction was >96% when total esterification was <60%,




**SCHEME 1**

**TABLE 1**
**Large-Scale Fractionation and Enrichment of CLA Isomers**

| Step | | Weight (g) | L-Menthol (g) | FFA (g) | L-Menthyl ester (g) |
|---|---|---|---|---|---|
| Esterification 1[a] | Before | 800 | 286 | 514 | 0 |
| | After | 725 | 145 | 260 | 320 |
| Distillation 1 | | | | | |
| Distillation 1-1[b] | | 133 | 132 | 1 | ND[c] |
| Distillate 1-mix[d] | | 300 | 10 | 202 | 88 |
| Residue 1-4[d] | | 276 | ND[c] | 45 | 231 |
| Esterification 2[e] | Before | 383 | 105 | 192 | 86 |
| | After | 355 | 65 | 120 | 170 |
| Distillation 2 | | | | | |
| Distillate 2-1[b] | | 55 | 54 | 1 | ND[c] |
| Distillate 2-mix[d] | | 158 | 4 | 107 | 47 |
| Residue 2-4[d] | | 122 | ND[c] | 8 | 114 |
| *n*-Hexane extraction | | | | | |
| Purified 10*t*,12*c*-CLA | | 108 | 3 | 105 | ND[c] |
| Esterification 3[f] | Before | 270 | 94 | 170 | 6 |
| | After | 242 | 30 | 53 | 159 |
| Distillation 3[b] | | | | | |
| Distillate 3-1 | | 26 | 26 | ND[c] | ND[c] |
| Residue 3-1 | | 207 | 2 | 49 | 156 |
| *n*-Hexane extraction | | 149 | 1 | 1 | 147 |
| Chemical hydrolysis[g] | | 143 | 39 | 101 | 3 |
| Distillation 4[b] | | | | | |
| Purified 9*c*,11*t*-CLA | | 99 | 2 | 96 | 1 |

[a]A mixture of 514 g FFA-CLA, 286 g L-menthol, 200 mL water, and $2.0 \times 10^5$ U *Candida rugosa* lipase was agitated at 30°C for 40 h.
[b]Distilled at 150°C and 270 Pa.
[c]Not detected, <0.2%.
[d]Distilled at 160, 175, and 190°C at 27 Pa, and all distillates were then mixed.
[e]A mixture of 287 g distillate 1-mix, 96 g L-menthol, 96 mL water, and $9.6 \times 10^4$ U *C. rugosa* lipase was agitated at 30°C for 40 h.
[f]After hydrolysis of residue 1-4 with NaOH, an equimolar mixture of the resulting FFA and L-menthol (270 g), 68 mL water, and $3.4 \times 10^4$ U *C. rugosa* lipase were agitated at 30°C for 40 h.
[g]Hydrolyzed with NaOH at 50°C for 40 min.

and the purity decreased slightly between 60 and 80% total esterification (Fig. 4). The purity and recovery were similar to those of selective esterification with lauryl alcohol (purity, 96%) (17) when total esterification was <60%. Hence, we set the conditions to achieve 60% total esterification as follows: FFA/L-menthol, 1:1 (mol/mol); water, 20 wt%; *C. rugosa* lipase, 100 U/g-mixture; temperature, 30°C; reaction period, 40 h. These conditions were applied to esterification of the FFA (19 g) that were prepared by hydrolysis of L-menthyl esters (total esterification, 61%). The subsequent *n*-hexane extraction recovered 15.6 g L-menthyl esters/L-menthol (recovery of 9*c*,11*t*-CLA, 54%), in which the purity of 9*c*,11*t*-CLA increased to 95%.

*Large-scale fractionation and enrichment of CLA isomers.* We attempted a large-scale fractionation of CLA isomers by a process comprising selective esterification and distillation. The processes are shown in Scheme 1, and the results are summarized in Table 1. A mixture containing 514 g FFA-CLA, 286 g L-menthol, 200 mL water, and 200 U/g-mixture of *C. rugosa* lipase was agitated at 30°C for 40 h (esterification 1; total esterification, 45%). The oil phase was fractionated into L-menthol, FFA, and L-menthyl ester fractions by distillation (distillation 1). L-Menthol was first removed into distillate 1 by simple distillation at 150°C and 270 Pa. The residue was then subjected to three-step short-path distillation and was fractionated into the mixture of three distillates (300 g; distillate 1-mix; FFA fraction) and residue 1-4 (276 g; L-menthyl ester fraction). The FA compositions of distillate 1-mix and residue 1-4 are shown in Table 2. The purities of 9*c*,11*t*- and 10*t*,12*c*-CLA were 84 and 86%, and their recoveries were 55 and 66%, respectively. If the purities of both isomers are high enough to be used as food supplements, we can reduce the cost by omitting the following processes. Furthermore, L-menthyl ester of 9*c*,11*t*-CLA may become a new food supplement as it is.

To further purify 10*t*,12*c*-CLA, the distillate 1-mix was esterified again. Although the distillate 1-mix contained 29 wt% L-menthyl esters (Table 1), the presence of the esters affected the enrichment of 10*t*,12*c*-CLA only slightly. The esterification of the distillate 1-mix was therefore performed at 30°C for 40 h by agitating a mixture of distillate 1-mix and an equimolar amount of L-menthol against FFA, 20 wt% water, and 200 U/g-mixture *C. rugosa* lipase (esterification 2; total esterification, 33%). The oil phase obtained from the reaction

**TABLE 2**
**FA Composition of the Fractionated and Enriched Products**

| | FA composition (wt%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CLA | | | | | | | | |
| Step | 9*c*,11*t* | 10*t*,12*c* | 9*c*,11*c* | 10*c*,12*c* | Others | 18:1 | 18:0 | 16:0 | Purity (%) |
| Original | 33.3 | 34.2 | 1.1 | 1.1 | 2.7 | 16.3 | 2.7 | 6.4 | — |
| Fractionation | | | | | | | | | |
| Distillate 1-mix | 11.4 | 57.8 | 1.9 | 2.1 | 4.6 | 10.1 | 4.6 | 7.5 | 83.5[a] |
| Residue 1-4 | 60.4 | 10.0 | 0.6 | 0.4 | 0.8 | 22.1 | 1.4 | 4.4 | 85.8[b] |
| Enrichment | | | | | | | | | |
| 10*t*,12*c*-CLA | 6.7 | 66.7 | 2.4 | 2.4 | 5.6 | 5.1 | 5.0 | 6.0 | 90.9[a] |
| 9*c*,11*t*-CLA | 74.2 | 4.5 | 0.2 | 0.2 | 0.4 | 18.4 | 0.4 | 1.7 | 94.3[b] |

[a]Purity of 10*t*,12*c*-CLA [= 10*t*,12*c*-CLA/(9*c*,11*t*-CLA + 10*t*,12*c*-CLA)].
[b]Purity of 9*c*,11*t*-CLA [= 9*c*,11*t*-CLA/(9*c*,11*t*-CLA + 10*t*,12*c*-CLA)].

mixture was subjected to distillation 2: Simple distillation removed L-menthol into distillate 2-1 (55 g), and three-step short-path distillation separated the residue into distillate 2-mix (158 g, mixture of three distillates, FFA fraction) and residue 2-4 (122 g). Because the distillate 2-mix contained 30% L-menthyl esters, the L-menthyl esters were removed by *n*-hexane extraction. The content of 10*t*,12*c*-CLA in the FFA fraction (108 g) increased from 34 to 67% (recovery, 40%), and the content of 9*c*,11*t*-CLA decreased to 6.7%, indicating that the purity of 10*t*,12*c*-CLA was 91% (Table 2). Little isomerization of 10*t*,12*c*-CLA occurred throughout the process. Furthermore, the purity was higher than that by selective hydrolysis (purity, 82%) (19) and that when using *G. candidum* lipase (purity, 81%) (16). Moreover, the recovery of 10*t*,12*c*-CLA was higher than that by selective hydrolysis of acylglycerols (recovery, 23%) (19) but was lower compared with that by selective esterification with lauryl alcohol (recovery, 52%) (17). As described previously, when FFA and L-menthyl esters were fractionated by *n*-hexane extraction, the recovery of 10*t*,12*c*-CLA was higher. Therefore, the low recovery was due to the low fractionation efficiency of short-path distillation, because the difference in M.W. between CLA L-menthyl esters (M.W., 418) and FFA (M.W., 280) is small.

9*c*,11*t*-CLA was subsequently purified from the L-menthyl ester fraction (residue 1-4) obtained by esterification 1. The residue (260 g) was chemically hydrolyzed with NaOH, and the FFA and L-menthol were recovered by *n*-hexane extraction (FFA/L-menthol = 1:0.85, mol/mol). L-Menthol was added to the mixture of FFA/L-menthol to give an equimolar amount against FFA. The mixture (270 g) was agitated at 30°C for 40 h with 100 U/g-mixture *C. rugosa* lipase in the presence of 20 wt% water (esterification 3; total esterification, 65%). The oil phase of the reaction mixture was then separated into an L-menthol fraction (26 g, distillate 3-1) and an FFA/L-menthyl ester fraction (207 g, residue 3-1) by simple distillation (distillation 3). Because the fractionation efficiency of FFA and L-menthyl esters by distillation 1 was low, the fractionation of FFA and L-menthyl esters in residue 3-1 was performed by *n*-hexane extraction. After the FFA that contaminated residue 3-1 were removed, the L-menthyl esters were hydrolyzed chemically, and the resulting FFA and L-menthol were recovered by *n*-hexane extraction. Finally, simple distillation of the FFA/L-menthol mixture at 150°C and 270 Pa (distillation 4) removed L-menthol into the distillate and recovered FFA in the residue (65 g). Little isomerization of either isomer of CLA occurred in this step, and the content of 9*c*,11*t*-CLA in the FFA fraction increased from 33 to 74% by a series of procedures (purity, 94%; recovery, 42%) (Table 2). The purity was high enough to produce nutraceuticals, although the process comprised several steps. The recovery was not only higher than that by selective hydrolysis (recovery, 33%) (19), but also higher than that by selective esterification with lauryl alcohol (recovery, 35%) (17). Haas *et al.* (15) achieved the purification of 9*c*,11*t*-CLA with very high purity (>98%) in an organic solvent by selective esterification with methanol or ethanol. However, in that case the recovery of the isomer was low because the reaction degree was low (≤22%).

*Features of the process developed in this study.* We developed a process that was effective for preparing high-purity 9*c*,11*t*- and 10*t*,12*c*-CLA from FFA-CLA in good yields compared with previous studies. The process comprised repeated esterifications with L-menthol using *C. rugosa* lipase, distillation, and *n*-hexane extraction; thus, it can be applied in the production of foods. In this research, distillation was adopted for the fractionation of FFA and L-menthyl esters, but the fractionation efficiency was lower than desired. The low efficiency and multistep fractionation may be drawbacks for industrialization. However, we were able to recover *ca.* 70% L-menthol in the large-scale fractionation. Therefore, the cost of fractionation will decrease if the L-menthol is repeatedly reused and optimization of the distillation conditions is achieved.

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