Fractionation and Enrichment of CLA Isomers by Selective Esterification with *Candida rugosa* Lipase

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ABSTRACT: A commercial product of CLA contains almost equal amounts of *cis*-9,*trans*-11 (*c*9,*t*11)-CLA and *trans*-10,*cis*-12 (*t*10,*c*12)-CLA. We attempted to enrich the two isomers by a two-step selective esterification using *Candida rugosa* lipase that acted on *c*9,*t*11-CLA more strongly than on *t*10,*c*12-CLA. An FFA mixture containing CLA isomers was esterified with an equimolar amount of lauryl alcohol in a mixture of 20% water and the lipase. When the esterification of total FA reached 50%, two isomers were fractionated in a good yield: *t*10,*c*12-CLA was enriched in FFA, and *c*9,*t*11-CLA was recovered in lauryl esters. The FFA were esterified again to enrich *t*10,*c*12-CLA. At 27.3% esterification of total FA, the *t*10,*c*12-CLA content in FFA increased to 64.8 wt% with 89.3% recovery: The ratio of the content of *t*10,*c*12-CLA to that of two isomers was 95.9%. Lauryl esters obtained by the single esterification were employed for enrichment of *c*9,*t*11-CLA. After the esters were hydrolyzed, the resulting FFA were esterified again with lauryl alcohol. At 62.0% esterification of total FA, the *c*9,*t*11-CLA content in lauryl esters increased to 73.3 wt% with 79.4% recovery: The ratio of the content of *c*9,*t*11-CLA to that of two isomers was 95.6%. In a 600-g-scale purification, molecular distillation was effective in separating the reaction mixture into lauryl alcohol, FFA, and lauryl ester fractions.

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KEY WORDS: *Candida rugosa*, conjugated linoleic acid, enrichment, lipase, molecular distillation, urea adduct fractionation.

CLA is a group of C18 FA containing a pair of conjugated double bonds in either the *cis* or *trans* configuration. It is industrially produced by alkali conjugation of safflower or sunflower oil in propylene glycol or ethylene glycol and contains almost equal amounts of *cis*-9,*trans*-11 (*c*9,*t*11)-CLA and *trans*-10,*cis*-12 (*t*10,*c*12)-CLA. The mixture of CLA isomers has various physiological activities, such as reduction of the incidence of cancer (1–3), decrease in body fat content (4,5), beneficial effects on atherosclerosis (6,7), and improvement of immune function (8). Natural CLA is present in meat and milk of ruminant animals (9), and the predominant isomer is *c*9,*t*11-CLA, which constitutes over 75% of total CLA (10,11). Although most of the physiological activities have been investigated using a mixture of CLA isomers, it was recently reported that *t*10,*c*12-CLA participates in the decrease of body fat (12–14) and that *c*9,*t*11-CLA possesses anticancer activity (15). These studies called a great deal of attention to the fractionation of CLA isomers.

Recently, the enzymatic method was found to be very effective for the fractionation of CLA isomers. Haas *et al*. (16) reported that *Geotrichum candidum* lipase recognized *c*9,*t*11-CLA more readily than *t*10,*c*12-CLA. They successfully enriched *c*9,*t*11-CLA in methyl esters from the products in the early stage of the reaction in which a mixture of CLA isomers was esterified with methanol in an organic solvent system. In addition, *c*9,*t*11-CLA was enriched in FFA from the products obtained by hydrolyzing methyl esters of CLA isomers. The purity of CLA isomers can be increased efficiently by their procedure, but the recovery is not good. At almost the same time, McNeill *et al*. (17) also indicated that *c*9,*t*11-CLA and *t*10,*c*12-CLA were fractionated with *G. candidum* lipase. They esterified a mixture of CLA isomers with lauryl alcohol and separated the reaction mixture into the FFA fraction (*t*10,*c*12-CLA rich) and lauryl ester fraction (*c*9,*t*11-CLA rich) by molecular distillation. The procedure allowed them to fractionate CLA isomers with a good recovery, but the purity was not high (80–85 wt%). In addition, *G. candidum* lipase is commercialized as a reagent, but is not suitable as a catalyst for oil processing because of low specific activity of the preparation. Our screening test showed that *Candida rugosa* lipase was also effective for the fractionation of CLA isomers. Hence, we attempted to fractionate *c*9,*t*11-CLA and *t*10,*c*12-CLA in a good yield and purity using *C. rugosa* lipase. This paper shows that a two-step process is effective for increasing the purity to >95% with a good yield.

MATERIALS AND METHODS

Materials. CLA was a commercial product (CLA-80; Rinoru Oil Mills Co. Ltd., Tokyo, Japan) obtained by alkali conjuga-

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tion of safflower oil in propylene glycol. The product contained 33.1 wt% *c*9,*t*11-CLA, 33.9 wt% *t*10,*c*12-CLA, 0.9 wt% *c*9,*c*11-CLA, and 1.4 wt% *c*10,*c*12-CLA, and 1.8 wt% other CLA isomers. Lauryl alcohol was purchased from Wako Pure Chemical Industry Co. (Osaka, Japan). *Candida rugosa* lipase was a gift from Meito Sangyo Co. (Aichi, Japan). One unit (U) of the activity was defined as the amount of enzyme that liberated 1 μmol FA/min in hydrolysis of olive oil (18).

Reaction conditions. A small-scale reaction was performed in a 50-mL vessel. A mixture of 4 g FFA/lauryl alcohol (1:1, mol/mol), 1 g water, and different amounts of *C. rugosa* lipase was incubated at 30°C for 16 h with stirring at 500 rpm. A large-scale reaction was performed at 30°C in a 2-L reactor (MDS-U; Marubishi Bioengineering Co. Ltd., Tokyo, Japan) with agitation at 350 rpm using the same composition of reactants as that of the small-scale reaction. The esterification degree of total FA was determined from the amount of FA consumed during the reaction. The esterification degree of a CLA isomer was expressed as a ratio of the amount of the isomer esterified to its initial amount.

Separation of lauryl esters and FFA in reaction mixture. A small-scale separation of lauryl esters and FFA was conducted by *n*-hexane extraction described previously (19). Lauryl esters and lauryl alcohol were extracted with 150 mL *n*-hexane after adding 70 mL 0.5 N KOH (20% ethanol solution) into 10 g reaction mixture. The FFA from the sodium salt in the water phase were extracted with 100 mL *n*-hexane after acidification with HCl.

A large-scale separation of lauryl alcohol, FFA, and lauryl esters was conducted with molecular distillation apparatus (Wiprene Type 2-03; Shinko Pantec Co. Ltd., Hyogo, Japan). Dehydration of the oil layer obtained after the lipase reaction was performed at 80°C and 3 mm Hg for 60 min with blowing nitrogen gas before applying molecular distillation. The water content was reduced to less than 100 ppm by the dehydration. Lauryl alcohol was distilled at 120°C and 0.2 mm Hg, and the residue was separated into the FFA fraction (distillate) and lauryl ester fraction (residue) by distilling at 190°C and 0.2 mm Hg.

Recovery of FFA from lauryl ester. Lauryl esters/lauryl alcohol mixture obtained by *n*-hexane extraction was first hydrolyzed with NaOH in the presence of ethanol. The reaction was conducted as follows: a mixture of 15 g lauryl ester fraction, 4.5 g NaOH, 6 mL water, and 114 mL ethanol was heated at 50°C for 30 min with blowing nitrogen gas. After the hydrolysis, 480 mL water was added to the reaction mixture, and lauryl alcohol was removed by extraction with 200 mL *n*-hexane. The FFA from the sodium salt in the water phase were then extracted with 200 mL *n*-hexane after acidification to pH 2 with HCl. The lauryl ester fraction recovered by molecular distillation was hydrolyzed on a threefold scale. After hydrolysis, 350 mL water was added to the reaction mixture, and FFA and lauryl alcohol were recovered by extraction with 350 mL *n*-hexane after acidification with HCl.

Urea adduct fractionation. Urea adduct fractionation was performed as described previously (20). In brief, a mixture of FFA/lauryl esters (50 g) was dissolved at 50°C in a solution of 250 mL methanol, 6.8 mL water, and 50 g urea, and the temperature was gradually decreased to 5°C with stirring. After removing the precipitate by filtration, 250 mL 0.2 N HCl was added to the resulting filtrate, and FFA were recovered with 400 mL *n*-hexane.

Analysis. Acid value was measured by titration with 0.5 to 1.0 N KOH. The molar amount of FFA was determined on the basis of the acid value. FFA were methylated in 3 mL 5% HCl/methanol by heating at 70°C for 10 min, and FA lauryl esters were converted to their methyl esters in 3 mL methanol containing 1% Na-methylate by heating at 70°C for 15 min. The FAME were analyzed with a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) connected to a DB-23 capillary 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 lauryl alcohol and lauryl esters were determined by GC with a DB-5 capillary column (0.25 mm × 10 m; J&W Scientific). The column temperature was raised from 150 to 300°C at 10°C/min, and was then maintained for 3 min. The injector and detector temperatures were 245 and 320°C, respectively. When a mixture of lauryl alcohol, FFA mixture containing CLA isomers, and lauryl esters (1:1:1, by wt) was analyzed under the above conditions, their peak areas were almost equal. Hence, the quantitative analysis was carried out based on their peak areas. In GC, the limit of detection was set at 0.15%.

RESULTS AND DISCUSSION

Determination of standard reaction conditions. Selective esterification of CLA isomers proceeded efficiently with one to two molar amounts of lauryl alcohol with respect to FFA. Since we previously found that greater than equimolar amounts of lauryl alcohol inhibited esterification of an FFA mixture containing arachidonic acid (21), the ratio of lauryl alcohol to FFA was fixed at 1:1 (mol/mol) in this study. When FFA mixture containing CLA isomers was esterified at 30°C for 16 h with an equimolar amount of lauryl alcohol using 20 U/g *C. rugosa* lipase in the presence of various amounts of water, the maximal esterification degree was achieved in the reaction mixture containing 20 to 30% water. We thus fixed the water content at 20%. Because the fractionation efficiency of CLA isomers depended only on the esterification degree of total FA, the reaction temperature and period were fixed at 30°C and 16 h, respectively.

Fractionation of CLA isomers by selective esterification. The FFA mixture containing CLA isomers was esterified at 30°C for 16 h with an equimolar amount of lauryl alcohol in a mixture containing 20% water and varying amounts of *C. rugosa* lipase. Figure 1A shows the relationship between the esterification degrees of each CLA isomer and total FA. *Candida rugosa* lipase acted on *c*9,*t*11-CLA more strongly than on *t*10,*c*12-CLA. Esterification of *c*9,*t*11-CLA reached a constant value (87%) at 50% esterification of total FA, and

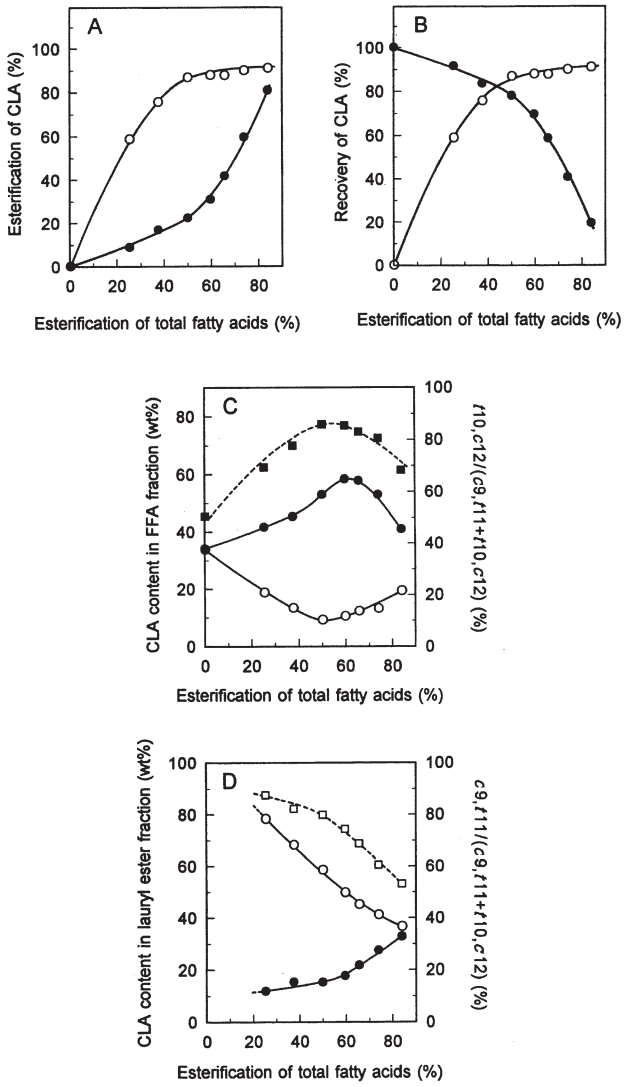


FIG. 1. Selective esterification of FFA mixture containing CLA isomers with lauryl alcohol using *Candida rugosa* lipase. A mixture of 2.4 g FFA, 1.6 g lauryl alcohol, and 1.0 g water was stirred at 30°C for 16 h using 4, 8, 20, 40, 80, 120, and 250 U/g-mixture of lipase. (A) Relationship between esterification degrees of *cis*-9,*trans*-11 (*c*9,*t*11)- and *trans*-10,*cis*-12 (*t*10,*c*12)-CLA isomers and that of total FA. (B) Recovery of CLA isomers. The recovery is expressed relative to the initial amount of each isomer. (C) Content of CLA isomers in the FFA fraction. (D) Content of CLA isomers in lauryl ester fraction. ○, *c*9,*t*11-CLA; ●, *t*10,*c*12-CLA; □, the ratio of the content of *c*9,*t*11-CLA to that of two isomers; ■, the ratio of the content of *t*10,*c*12-CLA to that of two isomers.

*t*10,*c*12-CLA was gradually esterified until 50% esterification of total FA and rapidly thereafter.

Figure 1B shows the recovery of *c*9,*t*11-CLA into the lauryl ester fraction and of *t*10,*c*12-CLA into the FFA fraction. Figures 1C and 1D show the contents of each CLA isomer in the FFA and lauryl ester fractions, respectively. The strong activity of *C. rugosa* lipase on *c*9,*t*11-CLA resulted in high *c*9,*t*11-CLA content in the lauryl ester fraction at low esterification of total FA (Fig. 1D), although the recovery into the ester fraction was low (Fig. 1B). The recovery of *c*9,*t*11-CLA increased with

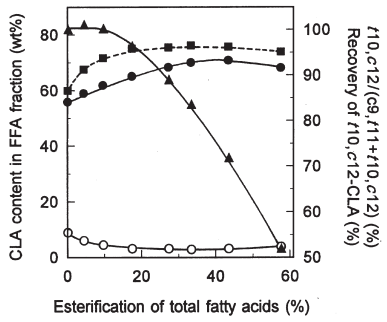


FIG. 2. Enrichment of *t*10,*c*12-CLA in the FFA fraction by repeated selective esterification. Unesterified FFA in the single reaction were recovered by extraction with *n*-hexane. A mixture of 2.4 g FFA, 1.6 g lauryl alcohol, and 1.0 g water was stirred at 30°C for 16 h using 4, 8, 20, 40, 80, 120, and 250 U/g-mixture of lipase. ○, Content of *c*9,*t*11-CLA in the FFA fraction; ●, content of *t*10,*c*12-CLA in the FFA fraction; ▲, recovery of *t*10,*c*12-CLA; ■, the ratio of the content of *t*10,*c*12-CLA to that of two isomers.

increasing degree of esterification of total FA, and its content in the ester fraction decreased. The recovery reached a constant value (87%) at 50% total esterification (Fig. 1B), and the content was 58 wt% (Fig. 1D). The ratio of the content of *c*9,*t*11-CLA to that of two isomers decreased as esterification of total FA increased, and the ratio was 79.5% at 50% esterification (Fig. 1D). The content of *t*10,*c*12-CLA in the FFA fraction reached the maximal value (58 wt%) at 60% total esterification (Fig. 1C), and the recovery significantly decreased as the esterification of total FA exceeded 50% (Fig. 1B). In addition, the ratio of the content of *t*10,*c*12-CLA to that of two isomers showed the maximal value (85.4%) at 50% esterification (Fig. 1C). These results indicated that the two isomers can efficiently be fractionated by ceasing the reaction at 50% esterification of total FA. Hence, the reaction conditions were set as follows: an

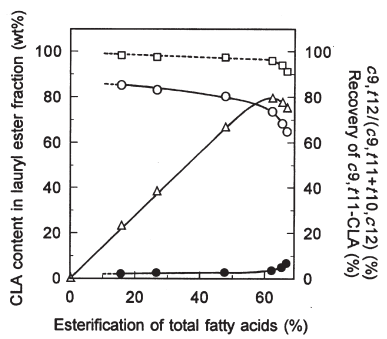
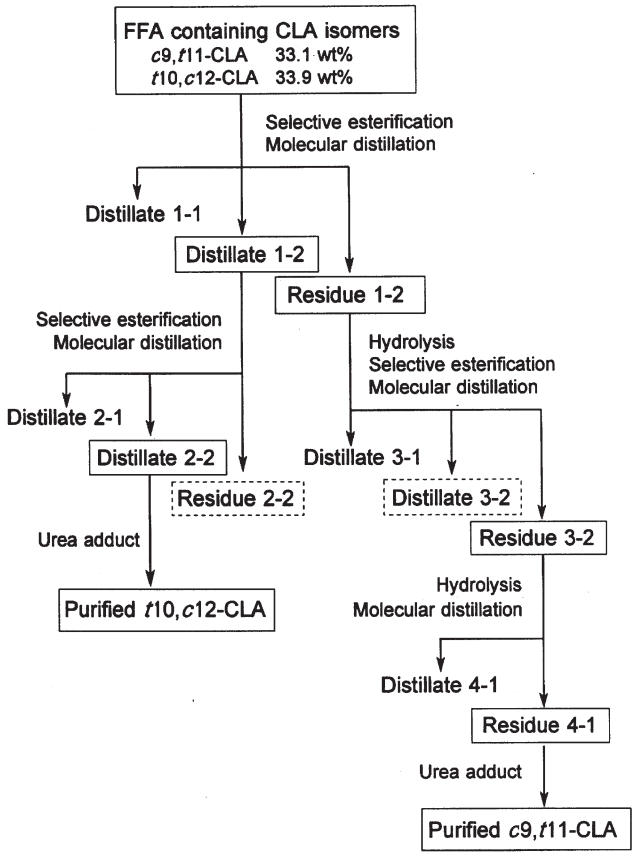


FIG. 3. Enrichment of *c*9,*t*11-CLA in the lauryl ester fraction by repeated selective esterification. Lauryl esters in the single reaction were recovered with *n*-hexane and were then hydrolyzed under alkaline conditions. The resulting FFA were esterified at 30°C for 16 h with an equimolar amount of lauryl alcohol in 5-g reaction mixture containing 20% water using 2, 4, 10, 20, 40, and 80 U/g-mixture of lipase. ○, Content of *c*9,*t*11-CLA in lauryl ester fraction; ●, content of *t*10,*c*12-CLA in lauryl ester fraction; △, recovery of *t*9,*c*11-CLA; □, the ratio of the content of *c*9,*t*11-CLA to that of two isomers.



SCHEME 1

equimolar amount of lauryl alcohol with respect to FFA, 20% water, 20 U/g-mixture of *C. rugosa* lipase, and incubation at 30°C for 16 h with stirring.

Enrichment of t*10,*c*12-CLA in FFA fraction by repeated esterification*. FFA containing CLA isomers were esterified under the conditions described above. In the unesterified FFA that were recovered with *n*-hexane after 50.2% esterification of total FA, *c*9,*t*11-CLA content was 9.0 wt%, and *t*10,*c*12-CLA content was 52.7 wt% (the ratio of the content of *t*10,*c*12-CLA to that of two isomers, 85.4%). The resulting FFA were esterified at 30°C for 16 h with an equimolar amount of lauryl alcohol in a mixture containing 20% water and various amounts of *C. rugosa* lipase. The *t*10,*c*12-CLA content in the FFA fraction reached the maximal value at 43% esterification of total FA, but the recovery into the FFA fraction decreased with increasing esterification (Fig. 2). When the esterification extent was 27.3%, the *t*10,*c*12-CLA content in the FFA fraction increased to 64.8 wt% with 89.3% recovery, and the *c*9,*t*11-CLA content decreased to 2.9 wt%. The content of *t*10,*c*12-CLA was 95.9% based on the total content of two isomers. These results showed that *t*10,*c*12-CLA can be enriched efficiently with 90% recovery by stopping the reaction at 25% total esterification. The reaction conditions were set as follows: an equimolar amount of lauryl alcohol with respect to FFA, 20% water, 40 U/g-mixture of *C. rugosa* lipase, and incubation at 30°C for 16 h with stirring.

Enrichment of c*9,*t*11-CLA in lauryl ester fraction by repeated esterification*. Lauryl esters were recovered with *n*-hexane after single selective esterification of FFA containing CLA isomers, which was the same reaction as that for fractionation of two isomers. The lauryl esters were hydrolyzed with NaOH in the presence of ethanol, and the resulting FFA were recovered with *n*-hexane after acidification with HCl to yield a product containing 58.4 wt% *c*9,*t*11-CLA and 15.1 wt% *t*10,*c*12-CLA. The content of *c*9,*t*11-CLA based on that of two isomers was 79.5%. The FFA were esterified at 30°C for 16 h with an equimolar amount of lauryl alcohol in a mixture containing 20% water and various amounts of *C. rugosa* lipase. The *c*9,*t*11-CLA content in the lauryl ester fraction gradually decreased with increasing esterification of total FA, and significantly decreased as esterification of total FA exceeded 60% (Fig. 3). Maximal recovery of *c*9,*t*11-CLA was obtained at 62% esterification of total FA. After the esterification, the *c*9,*t*11-CLA content increased to 73.3 wt% with 79.4% recovery, and the *t*10,*c*12-CLA content decreased to 3.4 wt%. The content of *c*9,*t*11-CLA was 95.6% based on the total content of the two isomers. To achieve 60% esterification of total FA, the reaction conditions were set as follows: an equimolar amount of lauryl alcohol with respect to FFA, 20% water, 20 U/g-mixture of *C. rugosa* lipase, and incubation at 30°C for 16 h with stirring.

Large-scale fractionation and enrichment of CLA isomers. We attempted a large-scale purification of *c*9,*t*11-CLA and *t*10,*c*12-CLA by a combination of selective esterification and distillation. The strategy is shown in Scheme 1, and the results are summarized in Table 1. FFA containing CLA isomers (600 g) were first esterified with an equimolar amount of lauryl alcohol with 20 U/g-mixture of *C. rugosa* lipase. Esterification of total FA reached 48.6% after 16 h. The oil layer was distilled at 120°C and 0.2 mm Hg, and lauryl alcohol was removed as distillate 1-1. The residue was next distilled at 190°C and 0.2 mm Hg, and the FFA fraction (distillate 1-2) and lauryl ester fraction (residue 1-2) were separated. The *t*10,*c*12-CLA content in the FFA fraction of distillate 1-2 was 55.6 wt%, and the *c*9,*t*11-CLA content in the lauryl ester fraction of residue 1-2 was 57.3 wt% (Table 2). The contents of *t*10,*c*12-CLA and *c*9,*t*11-CLA based on the total content of the two isomers were 86.4 and 81.9%, respectively.

To enrich *t*10,*c*12-CLA, selective esterification of the FFA fraction after the single reaction was attempted (Scheme 1 and Table 1). Distillate 1-2 contained 15.0 wt% lauryl esters, but the esters scarcely affected the reaction. Hence, distillate 1-2 was esterified with an equimolar amount (with respect to FFA) of lauryl alcohol using 40 U/g-mixture of *C. rugosa* lipase. The esterification reached 22.9% after 16 h, and the contents of *t*10,*c*12-CLA and *c*9,*t*11-CLA in the FFA fraction were 66.6 and 2.1 wt%, respectively. Lauryl alcohol in the reaction mixture was removed as distillate 2-1 by molecular distillation, and the FFA fraction was then recovered as distillate 2-2. Urea adduct fractionation of distillate 2-2 eliminated lauryl esters and saturated FA completely, and a part of the oleic acid. A series of procedures increased the *t*10,*c*12-CLA

TABLE 1
Large-Scale Fractionation and Enrichment of CLA Isomers

Step		Weight (g)	Acid value (mg KOH/g)	LauOH[a] (g)	FFA (g)	Lauryl ester (g)
Esterification 1[b]	Before	1000	129.1	399	601	0
	After	946	62.7	220	307	419
Distillate 1-1[c]		215	3.7	211	4	ND[d]
Distillate 1-2[e]		327	167.2	4	274	49
Residue 1-2[e]		371	10.2	ND[d]	19	352
Esterification 2[f]	Before	539	100.7	216	274	49
	After	511	77.6	127	199	185
Distillate 2-1[c]		128	4.9	125	3	ND[d]
Distillate 2-2[e]		193	169.2	2	164	27
Residue 2-2[e]		180	24.7	ND[d]	23	157
Urea adduct		136	198.2	1	135	ND[d]
Esterification 3[g]	Before	340	124.9	126	214	ND[d]
	After	328	48.7	29	80	219
Distillate 3-1[c]		27	8.9	26	1	ND[d]
Distillate 3-2[e]		98	133.3	2	66	30
Residue 3-2[e]		189	10.7	ND[d]	10	179
Hydrolysis[h]		170	115.3	66	100	4
Distillate 4-1[c]		63	3.2	62	1	ND[d]
Residue 4-1[c]		102	182.2	3	95	4
Urea adduct		86	197.4	2	84	ND[d]

[a]Lauryl alcohol.
[b]A mixture of FFA/lauryl alcohol (1:1, mol/mol) was incubated at 30°C for 16 h in a mixture containing 20% water using 20 U/g-mixture of *Candida rugosa* lipase.
[c]Distilled at 120°C and 0.2 mm Hg.
[d]Not detected, <0.15 wt%.
[e]Distilled at 190°C and 0.2 mm Hg.
[f]FFA (distillate 1-2)/lauryl alcohol (1:1, mol/mol) was incubated at 30°C for 16 h in a mixture containing 20% water using 40 U/g-mixture of *Candida* lipase.
[g]After hydrolysis of residue 1-2, the resulting FFA/lauryl alcohol (1:1, mol/mol) was incubated at 30°C for 16 h in a mixture containing 20% water using 20 U/g-mixture of *Candida* lipase.
[h]Residue 3-2 was hydrolyzed with NaOH.

content from 33.9 to 78.8 wt% with 52.3% recovery of its initial content and decreased the *c*9,*t*11-CLA content from 33.1 to 4.2 wt%; the *t*10,*c*12-CLA content was 94.9% based on the total content of two isomers (Table 2).

We next attempted to enrich *c*9,*t*11-CLA (Scheme 1 and Table 1). Lauryl esters (residue 1-2) obtained by the single esterification were hydrolyzed with NaOH in the presence of ethanol. After adding equal amounts of water and HCl to give pH 2, FFA and lauryl alcohol were recovered with *n*-hexane. Because the mixture contained almost equimolar amounts of FFA and lauryl alcohol, it was used as a substrate for enrichment of *c*9,*t*11-CLA. The reaction was performed at 30°C in a mixture containing 20% water and 20 U/g-mixture of *C. rugosa* lipase. The esterification extent of total FA reached 61.0% after 16 h, and the contents of *c*9,*t*11-CLA and *t*10,*c*12-CLA in the lauryl ester fraction were 75.1 and 3.4 wt%, respectively. After lauryl alcohol in the reaction mixture was removed as distillate 3-1 by molecular distillation, the residue was separated into distillate 3-2 (FFA fraction) and residue 3-2 (lauryl ester fraction). Residue 3-2 was hydrolyzed with NaOH in the presence of ethanol, and the resulting FFA and lauryl alcohol were recovered with *n*-hexane after acidification with HCl. The

TABLE 2
FA Composition in the Fractionated and Enriched Products[a]

	FA composition (wt%)							
				CLA				
Step	16:0	18:0	18:1	*c*9,*t*11	*t*10,*c*12	*c*9,*c*11	*c*10,*c*12	Others
Original	6.74	2.73	16.98	33.10	33.88	0.94	1.36	1.81
Fractionation								
Distillate 1-2	7.37	4.51	11.67	8.78	55.61	1.64	2.37	3.33
Residue 1-2	4.26	0.99	19.94	57.28	12.68	0.34	0.62	0.90
Enrichment								
*t*10,*c*12-CLA	ND	ND	2.51	4.19	78.78	2.27	2.42	4.85
*c*9,*t*11-CLA	ND	ND	9.54	83.48	3.81	0.29	0.32	0.72

[a]See Scheme 1; fractionated products are distillate 1-2 and residue 1-2, and enriched products are purified *trans (t)*10,*cis(c)*12-CLA and *c*9,*t*11-CLA.

mixture was separated to lauryl alcohol fraction (distillate 4-1) and FFA fraction (residue 4-1) by distillation. Because residue 4-1 contained 3.9 wt% lauryl esters and saturated FA, urea adduct fractionation was finally performed to remove them. These procedures increased the *c*9,*t*11-CLA content from 33.1 to 83.5 wt% with a 34.5% recovery of its initial content, and decreased the *t*10,*c*12-CLA content from 33.9 to 3.8 wt%; the *t*10,*c*12-CLA content was 95.6% based on the total content of two isomers (Table 2).

The results showed that *c*9,*t*11-CLA and *t*10,*c*12-CLA can be fractionated efficiently by the two-step procedure presented in this study. In addition, *C. rugosa* lipase as well as *G. candidum* lipase was found to be effective for the fractionation of the two isomers. In the large-scale purification, we did not make use of residue 2-2 (lauryl ester fraction after the reaction for enrichment of *t*10,*c*12-CLA) and distillate 3-2 (FFA fraction after the reaction for enrichment of *c*9,*t*11-CLA). The mixture of these fractions contained 31 wt% *c*9,*t*11-CLA and 29 wt% *t*10,*c*12-CLA of their initial contents, and their contents were *ca*. 30 wt%. Hence, the reuse of these fractions contributed to an increase in the recovery of CLA isomers.

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