

# Selective Enrichment of Conjugated Linoleic Acid Isomers in Their Mixtures Using Combined Chemical and Enzymatic Methods

Jiwon Kim<sup>1</sup> · Min-Yu Chung<sup>2</sup> · Hee-Don Choi<sup>2</sup> · In-Wook Choi<sup>2</sup> · In-Hwan Kim<sup>3</sup> · Hyang Sook Chun<sup>1</sup> · Byung Hee Kim<sup>4</sup>

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**Abstract** The aim of this study was to selectively enrich *t*10,*c*12-conjugated linoleic acid (*t*10,*c*12-CLA) and *c*9,*t*11-CLA in commercial CLA mixtures using a combination of urea crystallization and lipase-catalyzed esterification. The objective of the urea fractionation is to remove saturated and monounsaturated fatty acids (FA) from the CLA mixtures. CLA-enriched free FA (FFA) mixtures containing 53.8 wt% *t*10,*c*12-CLA and 39.1 wt% *c*9,*t*11-CLA were produced from the CLA mixtures containing ~34 wt% each of the two CLA isomers by a urea crystallization using methanol and the urea-to-FA weight ratio of 2.5:1. The CLA-enriched FFA mixtures were partially esterified with dodecan-1-ol in a recirculating packed-bed reactor using an immobilized lipase from *Candida rugosa* to further enrich the *t*10,*c*12-CLA and *c*9,*t*11-CLA in an FFA fraction and an FA dodecyl ester fraction, respectively, under the optimal conditions, i.e., temperature, 20 °C; FA-to-dodecan-1-ol molar ratio, 1:1; water content, 2 wt% of total substrates; residence time, 5 min; and reaction time, 24 h (for *t*10,*c*12-CLA enrichment) and 12 h (for *c*9,*t*11-CLA enrichment). After the reaction, an FFA fraction with 72.6 wt% *t*10,*c*12-CLA was obtained. Another FFA fraction with 62.0 wt% *c*9,*t*11-CLA was recovered after the saponification of the FA dodecyl ester fraction. The yields of *t*10,*c*12-CLA and

*c*9,*t*11-CLA in the FFA fractions were 43.6 and 21.5 wt%, respectively, based on their initial weights in the CLA mixtures.

**Keywords** Urea crystallization · Esterification · *Candida rugosa* lipase · Recirculating packed-bed reactor · *trans*-10,*cis*-12 Conjugated linoleic acid · *cis*-9,*trans*-11 Conjugated linoleic acid

## Introduction

“Conjugated linoleic acid” (CLA) means to a polyunsaturated fatty acids containing 18 carbons and two double bonds that are conjugated at positions  $\Delta 6/\Delta 8$ ,  $\Delta 7/\Delta 9$ ,  $\Delta 8/\Delta 10$ ,  $\Delta 9/\Delta 11$ ,  $\Delta 10/\Delta 12$ ,  $\Delta 11/\Delta 13$ , or  $\Delta 12/\Delta 14$  and can be in either the *cis* or the *trans* configuration [1]. Because four possible diastereomers (i.e., *cis*-*cis*, *cis*-*trans*, *trans*-*cis*, *trans*-*trans*) exist for each of the seven regioisomers, there are 28 isomers of CLA [2]. CLA is commercially produced by the alkali-catalyzed conjugation of edible oils (e.g., safflower oil) rich in linoleic acid (18:2n-6) containing two *cis* double bonds at positions  $\Delta 9$  and  $\Delta 12$  in propylene glycol [3]. *cis*-9,*trans*-11 (*c*9,*t*11)-CLA and *trans*-10,*cis*-12 (*t*10,*c*12)-CLA are the two predominant isomers found in commercial CLA products [4]. They are present at a ~1:1 ratio and constitute ~2/3 of the total fatty acids. Several animal studies and clinical trials on the biological activity of CLA have revealed that *t*10,*c*12-CLA is mainly responsible for the antiobesity properties of CLA, whereas the anticancer activity of CLA is primarily associated with *c*9,*t*11-CLA [5].

The utility of commercial CLA mixtures may be enhanced via selective enrichment of *t*10,*c*12-CLA or *c*9,*t*11-CLA in the mixtures [3]. In several published

✉ Byung Hee Kim  
bhkim@sookmyung.ac.kr

<sup>1</sup> Department of Food Science and Technology, Chung-Ang University, Anseong 17546, Korea

<sup>2</sup> Korea Food Research Institute, Seongnam 13539, Korea

<sup>3</sup> Department of Food and Nutrition, Korea University, Seoul 02841, Korea

<sup>4</sup> Department of Food and Nutrition, Sookmyung Women's University, Seoul 04310, Korea

studies, researchers have attempted to selectively enrich a targeted CLA isomer via lipase-catalyzed esterification of the CLA mixtures with an alcohol compound such as *L*-menthol [6] and dodecan-1-ol [7, 8]. In the published studies, *Candida rugosa* lipase has been employed as an effective biocatalyst for the esterification because this lipase selectively esterifies *c9,t11*-CLA containing a *cis*-9 double bond but shows no selectivity toward *t10,c12*-CLA without the *cis*-9 double bond. The reaction results in the enrichment of *t10,c12*-CLA in the free fatty acid (FFA) fraction and enrichment of *c9,t11*-CLA in the form of esters. The studies mentioned above involved stirred-batch reactor systems. Notably, the presence of considerable amounts of water (20–25 wt% based on the total substrates) is needed for the reaction to take place.

A packed-bed reactor (PBR) is the most commonly used reactor for immobilized lipase-catalyzed reactions because of the greater potential for reutilization of immobilized enzymes and higher reaction efficiency as compared to a stirred-batch reactor during reactions [9]. A recirculating PBR (RPBR) is a type of the reactor in which reaction mixtures eluted from the enzyme bed move back to the starting point repeatedly. A major advantage of an RPBR in a lipase-catalyzed reaction is that the reaction is allowed to have more time to proceed as compared to any conventional type of PBR.

Recently, we demonstrated that an RPBR system can be used for immobilized *C. rugosa* lipase-catalyzed esterification of commercial CLA mixtures with dodecan-1-ol for the selective enrichment of a targeted CLA isomer in the mixtures containing ~33 wt% each of the *t10,c12*-CLA and *c9,t11*-CLA [2]. We were able to obtain an FFA fraction containing ~55 wt% *t10,c12*-CLA after a reaction time of 36 h. The yield of *t10,c12*-CLA in the fraction was ~22 wt% based on its weight in the CLA mixtures. Nonetheless, enrichment of *c9,t11*-CLA has not been performed in the study.

Urea crystallization is a process for extraction of linear *n*-alkane from other hydrocarbons by including the *n*-alkane in the channels of the crystallized urea, thereby forming urea-*n*-alkane clathrates. The process can also be applied to separation of linear FA from FA that are less linear. Saturated FA or monounsaturated FA are more likely to form a complex with urea than polyunsaturated FA containing more *cis* double bonds than the former [10].

In the present study, our purpose was to enrich *t10,c12*-CLA and *c9,t11*-CLA, separately from the same reaction products. We also considered a new approach to further increase the concentration of CLA isomers in their concentrates. We utilized a combination of urea crystallization and lipase-catalyzed esterification for the enrichment process. Before the *C. rugosa* lipase-catalyzed esterification, urea crystallization of the CLA mixtures was performed to obtain CLA-enriched FFA mixtures where most of oleic

acid (18:1n-9) was removed because *C. rugosa* lipase shows selectivity toward the FA containing a *cis*-9 double bond. Then, a *t10,c12*-CLA-enriched FFA fraction and a *c9,t11*-CLA-enriched FFA fraction were prepared, respectively, from the CLA-enriched FFA mixtures via selective esterification of CLA isomers with dodecan-1-ol in the presence or absence of water using immobilized *C. rugosa* lipase, which preferentially esterifies *c9,t11*-CLA over *t10,c12*-CLA as the biocatalyst and an RPBR. The effects of water content and residence time on the concentration and yield of each CLA isomer in each FFA fraction were investigated.

## Materials and Methods

### Materials

Commercial CLA mixtures containing 34.2 wt% *t10,c12*-CLA, 33.5 wt% *c9,t11*-CLA, and 5.3 wt% other CLA isomers were acquired from HK Biotech Co. (Chinju, Korea). Urea (purity >99%) and dodecan-1-ol (purity >98%) were purchased from Samchun Pure Chemical Co. (Pyeongtaek, Korea). Immobilized *C. rugosa* lipase on Immobead 150 was acquired from Sigma Chemical Co. (St. Louis, MO, USA). The FA methyl ester (FAME) standards were purchased from Nu-Chek Prep, Inc. (Elysian, MN, USA) or Supelco (Bellefonte, PA, USA). All other reagents were of analytical grade.

### Urea Crystallization

The commercial CLA mixtures (50 g) were dissolved in methanol (500 mL) with the desired amount of urea (in urea-to-CLA mixture weight ratios of 1:1, 1.5:1, 2:1, 2.5:1, 3:1, and 3.5:1, respectively), and the resulting mixture was refluxed at 250 °C until urea was dissolved completely. The solution was cooled to ordinary temperature at a rate of approximately 2.5 °C/min and held at this temperature for 30 min. The solution was then incubated at 4 °C for 16 h to allow the urea-FFA complex to crystallize. The crystals were simply discarded by filtration through a Whatman No. 1 filter paper in a Buchner funnel. Methanol was then removed from the filtrate using a rotary vacuum evaporator at 40 °C, and the remaining filtrate was acidified by adding 300 mL of hot 0.1 N HCl. An uncrystallized portion of the CLA mixtures was recovered by extracting the acidified solution with 500 mL of *n*-hexane in a separatory funnel twice. The *n*-hexane layer was washed with 100 mL of water twice and dried over anhydrous sodium sulfate. The *n*-hexane was removed using a rotary vacuum evaporator at 40 °C to obtain the CLA-enriched FFA mixtures, which were then stored at –80 °C until use.

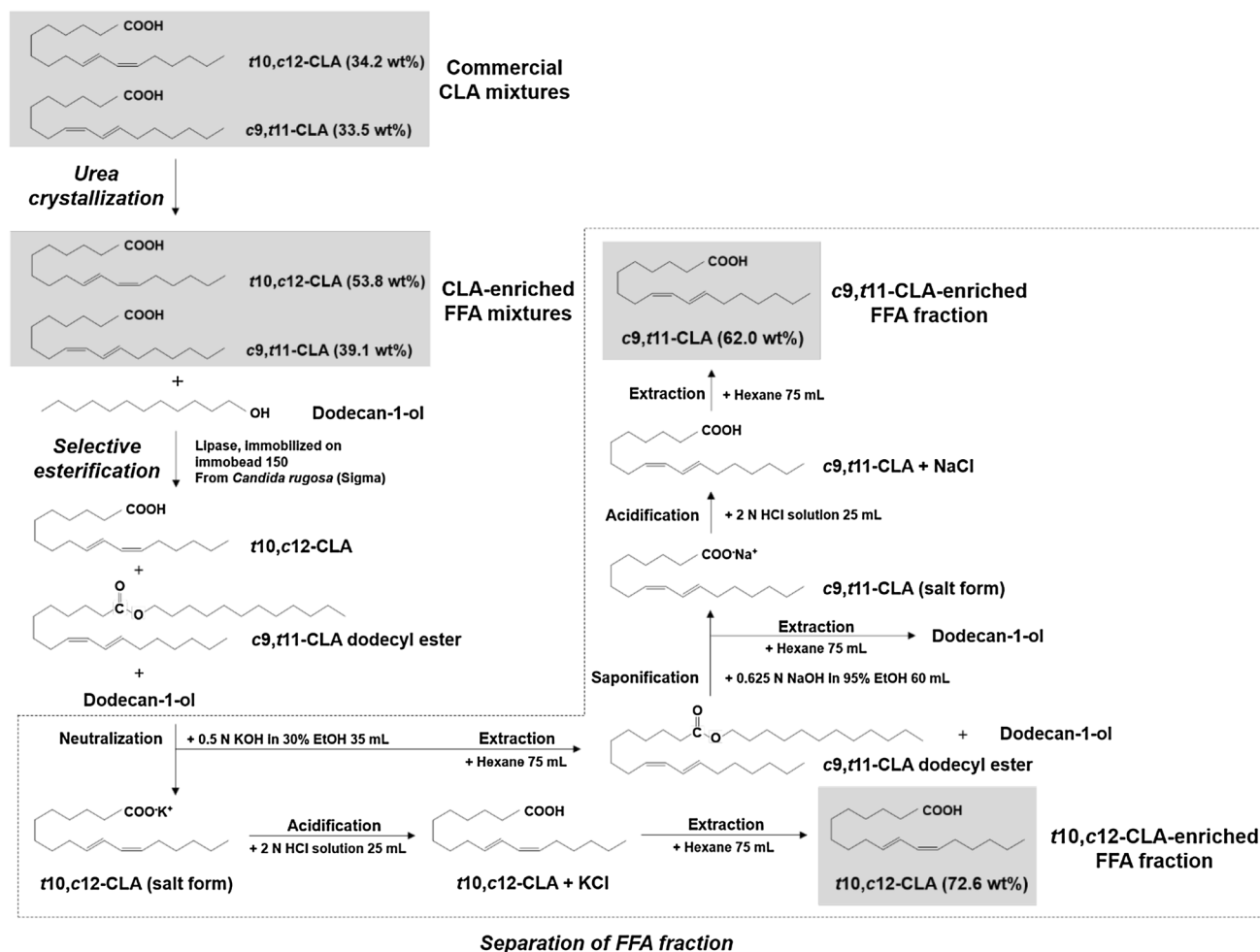
### C. rugosa Lipase-Catalyzed Esterification

The esterification of the CLA-enriched FFA mixtures with dodecan-1-ol was conducted in an RPBR consisting of a glass column (300 mm × 10 mm i.d.) equipped with a water jacket for temperature control. Immobilized *C. rugosa* lipase was used as the biocatalyst. The lipase (2 g) was manually packed into the column and was fixed in place using a stainless-steel sieve (50 mesh). The substrates contained CLA-enriched FFA mixtures and dodecan-1-ol in a molar ratio of 1:1. The substrates (80 g) were kept in a reservoir with a defined amount of added water (0, 2, 5, 10, 20, 40, and 60 wt% based on the total substrates). The reservoir consisted of a flat-bottom glass vessel (270 mm × 120 mm i.d.) with a water jacket for temperature control, and the substrates were agitated at 600 rpm using a magnetic stirrer during the reaction. The residence time of the substrates in the RPBR was 3, 5, 10, or 15 min. The RPBR and reservoir were maintained at 20 °C using

a water circulator (model RW-0252G, Jeio Tech, Seoul, Korea). The substrates were pumped into the column by a metering pump (model QG 150, Syosset, NY, USA). All trials were performed in duplicate.

### Separation of FFA from Reaction Products

A *t*10,*c*12-CLA-enriched FFA fraction and a *c*9,*t*11-CLA-enriched FFA fraction were separated, respectively, from the products obtained after the *C. rugosa* lipase-catalyzed esterification according to the method of Nagao *et al.* [7] with a slight modification, as depicted in Fig. 1. The reaction products (5 g) containing FFA (mainly, *t*10,*c*12-CLA), FA dodecyl esters (mainly, *c*9,*t*11-CLA dodecyl esters), and dodecan-1-ol were neutralized with 35 mL of 0.5 N KOH in 30 vol% ethanol at 35 °C for 30 min. The neutralized products were transferred into a 1-L separatory funnel and washed with 75 mL of *n*-hexane to extract FA dodecyl esters and dodecan-1-ol. The bottom aqueous layer was



**Fig. 1** Separation of free fatty acid fractions enriched in each CLA isomer from products obtained by *C. rugosa* lipase (immobilized on Immobead 150)-catalyzed esterification of the CLA-enriched FFA mixtures with dodecan-1-ol, after treatment by urea fractionation

collected and stirred with 25 mL of 2 N HCl to convert the potassium salts into their acid forms. Then, the layer was transferred in another 1-L separatory funnel and washed with 75 mL of *n*-hexane to extract a *t*10,*c*12-CLA-enriched FFA fraction. The FFA fraction was dried in a rotary vacuum evaporator and subsequently under a nitrogen flush.

To recover a *c*9,*t*11-CLA-enriched FFA fraction, the mixtures of FA dodecyl esters and dodecan-1-ol, which were extracted from the neutralized products mentioned above using 75 mL of *n*-hexane were saponified with 60 mL of 0.625 N NaOH in 95 vol% ethanol at 50 °C for 30 min. The saponified products were placed into a 1-L separatory funnel and was added with 140 mL of water to dissolve the FA sodium salts. Then, 75 mL of *n*-hexane was added to extract the dodecan-1-ol. The bottom aqueous layer was collected and stirred with 25 mL of 2 N HCl to convert the sodium salts into their acid forms. The layer was then placed in another 1-L separatory funnel and washed with 75 mL of *n*-hexane to extract a *c*9,*t*11-CLA-enriched FFA fraction. The FFA fraction was dried in a rotary vacuum evaporator and subsequently under a nitrogen flush.

### Analysis of Products

The relative amounts of FFA, FA dodecyl esters, and dodecan-1-ol in the FFA fractions were analyzed by gas chromatography (GC) by the method of Kang *et al.* [2]. An Agilent Technologies 7890A gas chromatograph (Palo Alto, CA, USA), equipped with a flame ionization detector (FID) and a fused silica capillary column (DB-17HT, 15 m × 0.25 mm i.d. × 0.15 μm film thickness, J&W Scientific, Folsom, CA, USA) was used for the analysis. Aliquots of the samples (25 mg) were dissolved in 1 mL of *n*-hexane, and 1 mL of the solution was injected in split mode with a split ratio of 50:1. The carrier gas was He, at a flow rate of 2.0 mL/min. The injector and detector temperatures were both 360 °C. The column was initially held at 120 °C for 1 min and was programmed to increase to 360 °C at a rate of 3 °C/min; it was then held at that temperature for 5 min.

The total FA compositions of CLA-enriched FFA mixtures, and FFA fractions were determined by the method of Kang *et al.* [11]. FFA samples (20 mg) were saponified with 3 mL of 0.5 N NaOH in methanol at 85 °C for 10 min, cooled to ordinary temperature, and then methylated with 3 mL of 14% BF<sub>3</sub> in methanol at 85 °C for 10 min. After cooling to ordinary temperature, 3 mL of isooctane and 5 mL of a saturated NaCl solution were added, and the mixture was vortexed. The upper isooctane layer containing FA methyl esters (FAME) was collected and passed through an anhydrous Na<sub>2</sub>SO<sub>4</sub> column. The FAME were analyzed by GC. An Agilent Technologies 7890A gas chromatograph, equipped with an FID and a fused silica capillary column (SP-2560,

100 m × 0.25 mm i.d. × 0.2 μm film thickness, Supelco) was used for the analysis. The sample (1 μL) was injected in split mode with a split ratio of 200:1. Helium gas served as the carrier gas at a flow rate of 1.0 mL/min. The injector and detector temperatures were set at 225 and 285 °C, respectively. The column was initially held at 100 °C for 4 min and was programmed to increase next to 240 °C at a rate of 3 °C/min; it was then held at that temperature for 17 min. The FAME were identified by comparing their retention time values with those of the standards, and their relative contents were calculated as wt%.

### Statistical Analysis

The differences in the FFA samples obtained by urea crystallization or *C. rugosa* lipase-catalyzed esterification were examined by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test. A value of *p* < 0.05 was taken to denote statistical significance.

## Results and Discussion

### Urea Crystallization of CLA Mixtures

Table 1 shows the FA composition of commercial CLA mixtures used as a starting material for the enrichment of targeted CLA isomers. They are mainly composed of two CLA isomers, *c*9,*t*11-CLA (33.5 wt%) and *t*10,*c*12-CLA (34.2 wt%), which constitute ~68 wt% of total FA. A considerable amount of oleic acid (14.6 wt%) was also found in the CLA mixtures. The FA profile of the CLA mixtures

**Table 1** Total FA composition of substrates (wt%)

Fatty acid	Commercial CLA mixtures	CLA-enriched FFA mixtures
14:0	0.1 ± 0.0 <sup>a</sup>	
16:0	7.6 ± 0.0	
16:1	0.1 ± 0.0	0.1 ± 0.0
18:0	2.7 ± 0.0	
18:1n-9	14.6 ± 0.0	3.0 ± 0.1
18:1n-7	0.7 ± 0.0	0.3 ± 0.0
18:2 <i>t</i>	0.5 ± 0.0	0.7 ± 0.0
18:2n-6	0.2 ± 0.0	0.6 ± 0.0
<i>c</i> 9, <i>t</i> 11-CLA	33.5 ± 0.1	39.1 ± 0.1
<i>t</i> 10, <i>c</i> 12-CLA	34.2 ± 0.1	53.8 ± 0.3
Other CLA isomers	5.3 ± 0.3	2.4 ± 0.2
20:0	0.3 ± 0.0	
20:1	0.1 ± 0.0	
Unidentified	0.1 ± 0.0	

<sup>a</sup> Mean ± standard deviation (*n* = 2)

revealed that if the CLA mixtures are directly esterified with dodecan-1-ol using *C. rugosa* lipase as the biocatalyst, the alcohol will be esterified with oleic acid as well as *c9,t11*-CLA, thereby resulting in the enrichment of both FA in the form of dodecyl esters. Eventually, this process can lead to a reduction in the relative content of *c9,t11*-CLA in the FFA fraction recovered from the FA dodecyl esters.

Urea crystallization can be applied to separation of oleic acid from *c9,t11*-CLA or *t10,c12*-CLA because oleic acid is more linear and more saturated than the two CLA isomers. Accordingly, before the *C. rugosa* lipase-catalyzed esterification, urea crystallization of commercial CLA mixtures was performed to obtain an FFA fraction from which oleic acid as well as saturated FA such as palmitic (16:0) and stearic acids (18:0) were removed. Urea-to-CLA mixture weight ratios of 1:1–3.5:1 were tested in the present study, with methanol serving as the solvent. Figure 2 shows variations in the oleic acid, *c9,t11*-CLA, and *t10,c12*-CLA content and the yield of the two CLA isomers. As the urea-to-CLA mixture weight ratios increased from 1:1 to 3.5:1, the oleic acid content decreased and *t10,c12*-CLA content increased in the CLA-enriched FFA mixtures. Unlike *t10,c12*-CLA, *c9,t11*-CLA showed a maximal content value at a ratio of 2:1 or 2.5:1. The yield of both CLA isomers steadily decreased with the increase in the ratio. These results are in agreement with our expectations that the three FA are removed in the order of oleic acid, *c9,t11*-CLA, and *t10,c12*-CLA, by forming a complex with urea. This result arises from the fact that the length of the linear hydrocarbon chain is greater in *c9,t11*-CLA than in *t10,c12*-CLA—i.e., *c9,t11*-CLA contains the *cis* double bond between carbons 9 and 10, when counted from the methyl end of the hydrocarbon chain, whereas *t10,c12*-CLA has the double bond between carbons 6 and 7. Thus, the urea-to-CLA mixture weight ratio of 2.5:1 was found to be the best for minimizing the level of remaining oleic acid while maximizing

*c9,t11*-CLA, and *t10,c12*-CLA content. The FA composition of CLA-enriched FFA mixtures at the ratio of 2.5:1 is also given in Table 1. In the CLA-enriched FFA mixtures, the oleic acid content decreased from 14.6 wt% (in the commercial CLA mixtures) to 3.0 wt%. The table shows that the urea crystallization process afforded CLA-enriched FFA mixtures containing 39.1 wt% *c9,t11*-CLA and 53.8 wt% *t10,c12*-CLA with a yield of 42.2 wt% for *c9,t11*-CLA and 57.2 wt% for *t10,c12*-CLA. The CLA-enriched FFA mixtures did not contain any saturated FA such as palmitic, stearic, or arachidic acids (20:0), which were originally present in the commercial CLA mixtures.

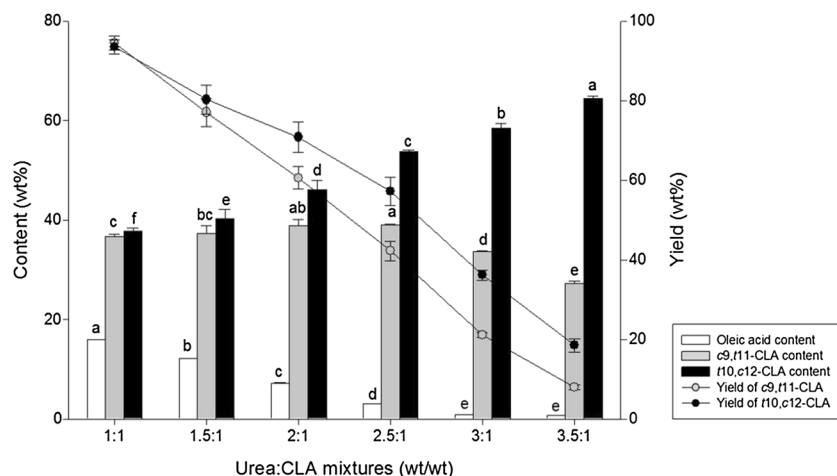
### *C. rugosa* Lipase-Catalyzed Esterification

After the urea crystallization, the CLA-enriched FFA mixtures were esterified with dodecan-1-ol using immobilized *C. rugosa* lipase as the biocatalyst. This reaction enables enrichment of *t10,c12*-CLA and *c9,t11*-CLA in an FFA fraction and an FA dodecyl ester fraction, respectively, because this lipase selectively esterifies *c9,t11*-CLA with dodecan-1-ol. An RPBR system was used for the reaction. We evaluated the effects of two key reaction parameters, namely, water content and residence time, on the enrichment of *t10,c12*-CLA and *c9,t11*-CLA. Because several published studies including ours [2, 7, 8] revealed that the optimal temperature and FFA-to-dodecan-1-ol molar ratio for the *C. rugosa* lipase-catalyzed esterification with dodecan-1-ol are near the ordinary temperature (i.e., 20–30 °C) and 1:1, respectively, the effects of temperature and substrate molar ratio were not assessed in the present study.

### Effects of Water Content

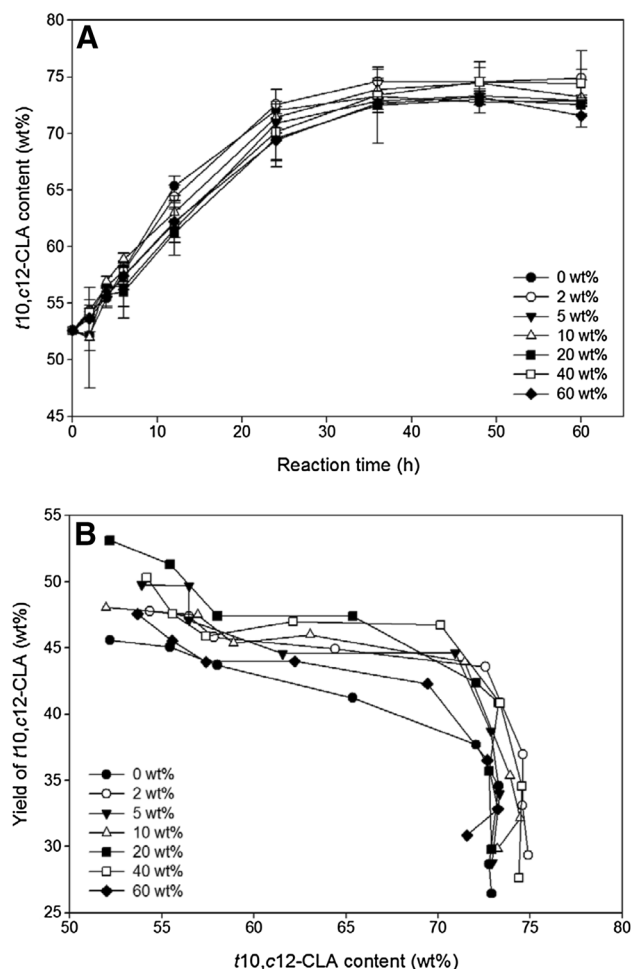
The proportion of water in the substrates is one of the crucial factors affecting the lipase activity and stability during

**Fig. 2** Effects of the amount of urea on the content and the yield of *t10,c12*-CLA and *c9,t11*-CLA in the CLA-enriched FFA mixtures obtained by urea crystallization of the commercial CLA mixtures. Means with different letters for a given FFA type are significantly different ( $p < 0.05$ ). The CLA mixtures contained 14.6 wt% oleic acid, 33.5 wt% *c9,t11*-CLA, and 34.2 wt% *t10,c12*-CLA



lipase-catalyzed esterification [12]. A minimal amount of water is required to maintain the specific three-dimensional structure of lipase, whereas addition of a large quantity of water to the reaction system can decrease lipase activity or shift the reaction toward hydrolysis [13]. Nevertheless, Kobayashi *et al.* [6] and Nagao *et al.* [7, 8] reported that the selective esterification of CLA isomers catalyzed by *C. rugosa* lipase takes place efficiently in the presence of a relatively large amount of water (20–25 wt% of the total substrates). A recent finding by No *et al.* [14]—who used the *C. rugosa* lipase-catalyzed esterification for the selective enrichment of pinolenic acid in pine nut oil—support these previous results. They also utilized a stirred-batch reactor system to carry out the reaction and reported that the optimal level of added water is 10 wt% of the total substrates in the reaction system, whereas the reaction does not proceed without added water. In contrast, according to our previous study [2], which involved the same type of reactor and enzyme as that used in the current study, little or no added water (e.g., 2 wt% of the total substrates) was needed for the esterification reaction, although a small amount of water is also formed during the reaction and is likely to accumulate in the reaction mixtures. These conflicting results show the necessity of independent studies on the effects of the amount of added water on the esterification reaction in our RPBR system.

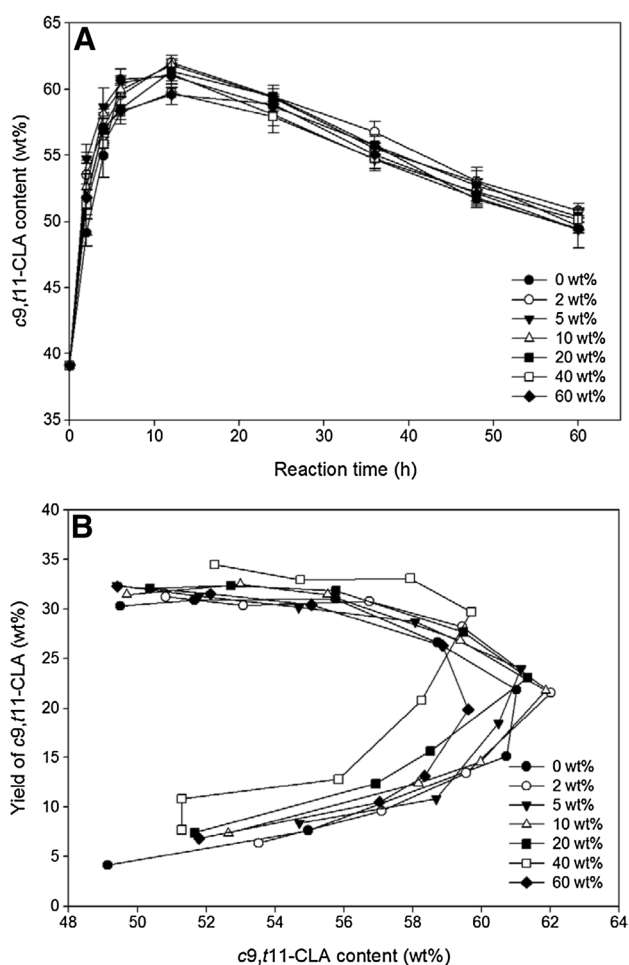
Figure 3a shows the effects of water content of the substrates on the  $t_{10,c12}$ -CLA content of the FFA fraction as a function of reaction time. Water content varied from 0 to 60 wt%. For these trials, the temperature, molar ratio of CLA-enriched FFA mixtures to dodecan-1-ol, and residence time of the substrates in the RPBR were maintained at 20 °C, 1:1, and 5 min, respectively.  $t_{10,c12}$ -CLA content in all trials increased with reaction time but tended to reach a plateau (at approximately 72–75 wt%) after 36 h. There was no significant difference in  $t_{10,c12}$ -CLA content among all the trials throughout the entire reaction period, except for two time points: 12 and 60 h—e.g., at 60 h, the  $t_{10,c12}$ -CLA content in the trial with water content of 2 wt% was significantly greater than that of the trial with water content at 60 wt%. These results suggest that in our reaction system based on an RPBR, the *C. rugosa* lipase-catalyzed esterification can efficiently proceed regardless of the amount of added water. Just as in our previous study [2], these results contradict other studies [6–8, 14]. The reason for the conflicting results is still unclear; however, one thing which is clearly different from other studies is that our reaction system involves a PBR in contrast to their use of a stirred-batch reactor system. To select optimal water content for preparing  $t_{10,c12}$ -CLA-enriched FFA fractions, a correlation between  $t_{10,c12}$ -CLA content and the yield of  $t_{10,c12}$ -CLA at different water content values is presented in Fig. 3b. The curve closer to the upper-right corner of the graph suggests that greater  $t_{10,c12}$ -CLA content can be



**Fig. 3** Effects of water content on the  $t_{10,c12}$ -CLA content (a) and the correlation between the  $t_{10,c12}$ -CLA content and the yield of  $t_{10,c12}$ -CLA (b) in the free fatty acid fraction produced by *C. rugosa* lipase (immobilized on Immobead 150)-catalyzed esterification of the CLA-enriched FFA mixtures with dodecan-1-ol as a function of reaction time (0, 2, 4, 6, 12, 24, 36, 48, and 60 h). The yield of  $t_{10,c12}$ -CLA was calculated based on the initial weight of the  $t_{10,c12}$ -CLA in the commercial CLA mixtures. The reaction was performed at 20 °C, a substrate molar ratio of 1:1 (CLA-enriched FFA mixtures to dodecan-1-ol), and a residence time of 5 min in a recirculating packed bed reactor. The weights of the total substrates and the enzyme loading were 80 and 2 g, respectively

achieved at a given yield of  $t_{10,c12}$ -CLA or a greater yield of  $t_{10,c12}$ -CLA can be obtained at a desired  $t_{10,c12}$ -CLA content. The curve obtained from the trial with water content of 40 wt% was closer to the upper-right corner of the graph than the others at  $t_{10,c12}$ -CLA content of less than ~70 wt%. Nevertheless, with  $t_{10,c12}$ -CLA content greater than ~70 wt%, the curve obtained from the trial with water content of 2 wt% was closer to the upper-right corner of the graph as compared to that obtained from the trial with water content of 40 wt%. Consequently, water content of 2 wt% was the most desirable for the  $t_{10,c12}$ -CLA enrichment.

Figure 4a illustrates the effects of water content of the substrates on *c9,t11*-CLA content of the FFA fraction as a function of reaction time. All conditions were the same as those listed in Fig. 3. Unlike the *t10,c12*-CLA content reaching a plateau after a certain period of reaction time, *c9,t11*-CLA content in all trials increased to a maximal value of ~58 to 62 wt% with longer reaction time during the first 12 h of the reaction, but decreased with reaction time after 12 h. These results arise from the fact that *C. rugosa* lipase also esterifies other FA without a *cis*-9 double bond (including *t10,c12*-CLA) with dodecan-1-ol as *c9,t11*-CLA is gradually depleted. After *c9,t11*-CLA content reached a maximal value at 12 h, no significant difference was observed in *c9,t11*-CLA content among all trials.



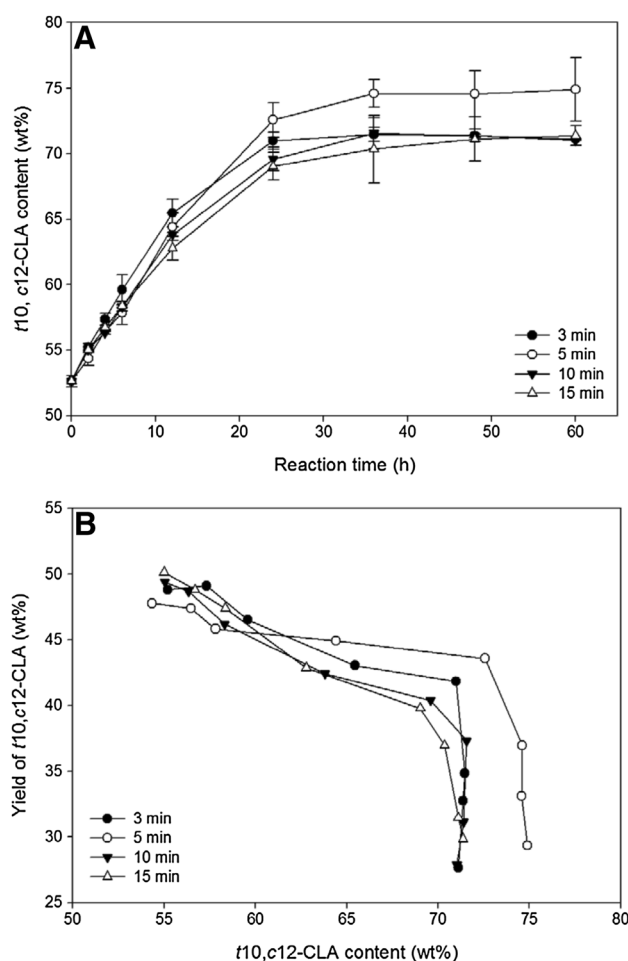
**Fig. 4** Effects of water content on the *c9,t11*-CLA content (a) and the correlation between the *c9,t11*-CLA content and the yield of *c9,t11*-CLA (b) in the free fatty acid fraction obtained from the fatty acid dodecyl ester fraction produced by *C. rugosa* lipase (immobilized on Immobead 150)-catalyzed esterification of the CLA-enriched FFA mixtures with dodecan-1-ol as a function of reaction time. The yield of *c9,t11*-CLA was calculated based on the initial weight of the *c9,t11*-CLA in the commercial CLA mixtures. The reaction was performed under the conditions given in Fig. 3

These results also clearly show that water content of the substrates does not markedly affect the efficiency of the *C. rugosa* lipase-catalyzed esterification for *c9,t11*-CLA enrichment. Figure 4b depicts a correlation between *c9,t11*-CLA content and the yield of *c9,t11*-CLA at different water content values. With a *c9,t11*-CLA content below ~60 wt%, the curve acquired from the trial with water content of 40 wt% was closer to the upper-right corner of the graph than the others, whereas the curve obtained from the trial with water content of 2 wt% was closer to the upper-right corner of the graph as compared to that obtained from the trial with water content of 40 wt% when the *c9,t11*-CLA content was greater than ~60 wt%. Therefore, the optimal water content of the substrates for the *c9,t11*-CLA enrichment was found to be 2 wt% of total substrates, same as that for the *t10,c12*-CLA enrichment.

#### Effects of Residence Time

The enzyme-to-substrate ratio greatly affects the efficiency of an enzyme-catalyzed reaction [15]. In a PBR system, because the amount of the enzyme used in the system is fixed, the enzyme-to-substrate ratio can be adjusted only by controlling the residence time, which is the time that substrates spend in the enzyme bed. Residence time is calculated by means of the following equation: residence time (min) = void volume of enzyme bed (mL)/flow rate of substrates (mL/min). In our RPBR system, the residence time can be controlled by adjusting the flow rate of the substrates fed into the immobilized *C. rugosa* lipase-packed bed column.

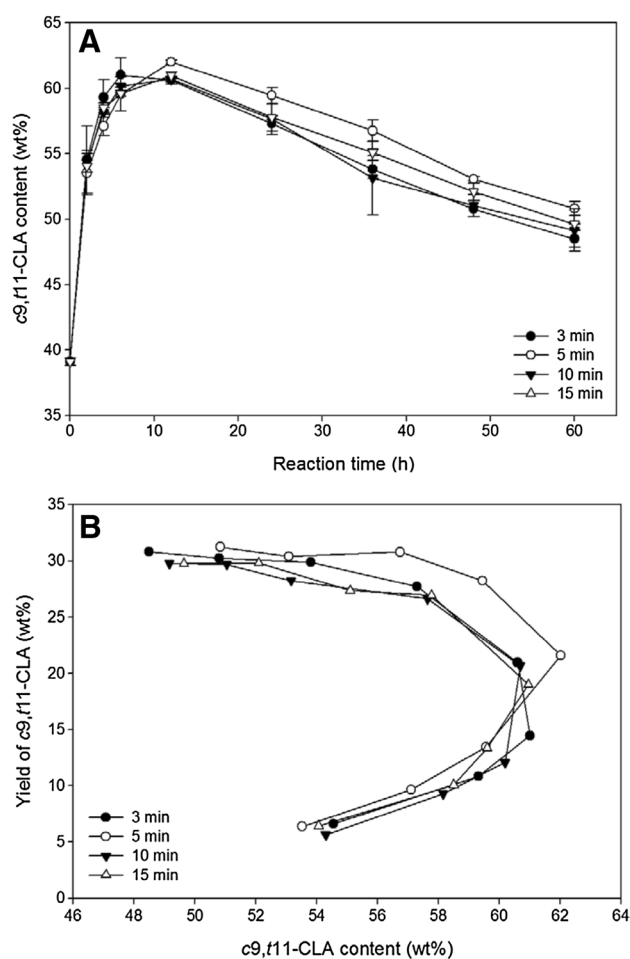
Figure 5a shows the effects of residence time on *t10,c12*-CLA content of the FFA fraction as a function of reaction time. The residence time tested ranged from 3 to 15 min. In these trials, the temperature, molar ratio of CLA-enriched FFA mixtures to dodecan-1-ol, and water content of the substrates in the RPBR were kept constant at 20 °C, 1:1, and 2 wt%, respectively. No remarkable difference was observed in *t10,c12*-CLA content among all trials during the first 24 h of the reaction. By contrast, after 36 h, the *t10,c12*-CLA content tended to be greater in the trial with residence time of 5 min than in the others although the difference did not reach statistical significance in some comparisons. A correlation between *t10,c12*-CLA content and the yield of *t10,c12*-CLA at different residence time values is presented in Fig. 5b. The curve obtained from the trial with residence time at 5 min was closer to the upper-right corner of the graph than the others, indicating that the optimal residence time for the *t10,c12*-CLA enrichment is 5 min. These results are in agreement with the findings by others. For instance, Watanabe *et al.* [16] found that shorter residence time is more desirable for the lipase-catalyzed direct esterification of glycerol and FA for



**Fig. 5** Effects of residence time on the  $t_{10},c_{12}$ -CLA content (a) and the correlation between the  $t_{10},c_{12}$ -CLA content and the yield of  $t_{10},c_{12}$ -CLA (b) in the free fatty acid fraction produced by *C. rugosa* lipase (immobilized on Immobead 150)-catalyzed esterification of the CLA-enriched FFA mixtures with dodecan-1-ol as a function of reaction time. The yield of  $t_{10},c_{12}$ -CLA was calculated based on the initial weight of the  $t_{10},c_{12}$ -CLA in the commercial CLA mixtures. The reaction was performed at 20 °C, a substrate molar ratio of 1:1 (CLA-enriched FFA mixtures to dodecan-1-ol), and a water content of 2 wt% of total substrates in a recirculating packed bed reactor. The weights of the total substrates and the enzyme loading were 80 and 2 g, respectively

the diacylglycerol production in an RPBR. Zhao *et al.* [17] also reported that relatively short residence time of 3 min is optimal for lipase-catalyzed ethanolsis of pine nut oil to obtain a pinolenic acid concentrate in the FA ethyl ester form when an RPBR is employed.

Figure 6a presents the effects of residence time on  $c_{9},t_{11}$ -CLA content of the FFA fraction as a function of reaction time. All conditions were the same as those given in Fig. 5. After 12 h,  $c_{9},t_{11}$ -CLA content tended to be greater in the trial with residence time of 5 min than in the others although the difference did not reach statistical significance in some comparisons. Figure 6b (illustrating



**Fig. 6** Effects of residence time on the  $c_{9},t_{11}$ -CLA content (a) and the correlation between the  $c_{9},t_{11}$ -CLA content and the yield of  $c_{9},t_{11}$ -CLA (b) in the free fatty acid fraction obtained from the fatty acid dodecyl ester fraction produced by *C. rugosa* lipase (immobilized on Immobead 150)-catalyzed esterification of the CLA-enriched FFA mixtures with dodecan-1-ol as a function of reaction time. The yield of  $c_{9},t_{11}$ -CLA was calculated based on the initial weight of the  $c_{9},t_{11}$ -CLA in the commercial CLA mixtures. The reaction was performed under the conditions given in Fig. 5

a correlation between  $c_{9},t_{11}$ -CLA content and the yield of  $c_{9},t_{11}$ -CLA at different residence time values) also indicates that the optimal residence time for the  $c_{9},t_{11}$ -CLA enrichment is 5 min, same as that for the  $t_{10},c_{12}$ -CLA enrichment.

#### Enrichment of CLA Isomers Under Optimal Conditions

In Figs. 3b and 5b, the points closest to the upper-right corner of the graph were obtained at 24 h, indicating that the optimal reaction time is 24 h for the  $t_{10},c_{12}$ -CLA enrichment. Using the same method, the optimal reaction time was found to be 12 h for the  $c_{9},t_{11}$ -CLA enrichment (Figs. 4b, 6b). Accordingly, the optimal conditions



include a temperature of 20 °C, FA-to-dodecan-1-ol molar ratio of 1:1, water content of 2 wt% of total substrates, residence time of 5 min, and reaction time of 24 h (for *t10,c12*-CLA enrichment) and 12 h (for *c9,t11*-CLA enrichment). From the reaction products obtained under the optimal conditions for the *t10,c12*-CLA enrichment, an FFA fraction with 72.6 wt% *t10,c12*-CLA can be obtained. Another FFA fraction with 59.4 wt% *c9,t11*-CLA can also be recovered from the same reaction products. The yield of *t10,c12*-CLA and *c9,t11*-CLA was 43.6 and 28.2 wt% of their initial weights, respectively, in the commercial CLA mixtures.

An FFA fraction with 62.0 wt% *c9,t11*-CLA can be recovered from the reaction products obtained under the optimal conditions for the *c9,t11*-CLA enrichment. Another FFA fraction with 64.4 wt% *t10,c12*-CLA can also be obtained from the same reaction products. The yield of *c9,t11*-CLA and *t10,c12*-CLA was 21.5 and 44.9 wt% of their initial weights, respectively, in the commercial CLA mixtures.

## Conclusion

Selective enrichment of *t10,c12*-CLA and *c9,t11*-CLA from commercial CLA mixtures was successfully achieved using a combination of urea crystallization and *C. rugosa* lipase-catalyzed esterification under the optimal conditions that we identified. We prepared CLA-enriched FFA mixtures with a reduced concentration of oleic acid (3.0 wt%) from the commercial CLA mixtures containing 14.6 wt% oleic acid using a urea crystallization method. Because *C. rugosa* lipase is selective toward the FA containing a *cis*-9 double bond, the CLA-enriched FFA mixtures can serve as good substrates for the *C. rugosa* lipase-catalyzed selective esterification of CLA isomers. The esterification was carried out in an RPBR. The optimal conditions were the following: temperature, 20 °C; FA-to-dodecan-1-ol molar ratio, 1:1, water content, 2 wt% of total substrates; residence time, 5 min; and reaction time, 24 h (for *t10,c12*-CLA enrichment) and 12 h (for *c9,t11*-CLA enrichment). Under these conditions, we obtained an FFA fraction containing 72.6 wt% *t10,c12*-CLA and another FFA fraction with *c9,t11*-CLA content of 62.0 wt%. The yield of *t10,c12*-CLA and *c9,t11*-CLA in the FFA fractions were 43.6 and 21.5 wt% of their initial weights, respectively, in the commercial CLA mixtures.

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