Enzymatic Fractionation and Enrichment of n-9 PUFA

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ABSTRACT: A single-cell oil from a *Mortierella alpina* mutant (TGM17 oil) contains n-9 PUFA: 14.3 wt% 6,9-octadecadienoic acid (18:2n-9; n-9 LnA) and 17.1 wt% Mead acid (20:3n-9; MA). Lipase screening indicated that *Pseudomonas aeruginosa* lipase acted strongly on n-9 LnA and weakly on MA, and *Candida rugosa* lipase acted weakly on the two PUFA. Hence, fractionation and enrichment of the two FA were conducted with the lipases. The first step was selective hydrolysis of TGM17 oil with *P. aeruginosa* lipase. The hydrolysis fractionated the oil into FFA containing 20.4 wt% n-9 LnA and 6.3 wt% MA, and acylglycerols containing 10.7 wt% n-9 LnA and 23.7 wt% MA. The FFA fraction was used for preparation of n-9 LnA-rich FFA. After removal of saturated FA, the FFA were esterified with lauryl alcohol (LauOH) using *C. rugosa* lipase. Two selective esterifications increased the n-9 LnA content to 54.0 wt% with 38.2% recovery of the initial content of TGM17 oil. The acylglycerol fraction obtained in the hydrolysis with *P. aeruginosa* lipase was used for preparation of MA-rich FFA. The acylglycerol fraction was hydrolyzed under alkaline conditions, and saturated FA were eliminated by urea adduct fractionation. Two selective esterifications of the FFA with LauOH increased the MA content to 60.2 wt% with 53.5% recovery. Thus, the two-step enzymatic process was effective for fractionation and enrichment of n-9 LnA and MA.

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KEY WORDS: *Candida rugosa*, esterification, hydrolysis, lipase, Mead acid, *Mortierella alpina*, n-9 linoleic acid, PUFA, *Pseudomonas aeruginosa*, single-cell oil.

Mead acid (20:3n-9; MA) is an unusual FA that was first isolated from EFA-deficient rats in 1956 (1). MA inhibited the synthesis of leukotriene B_4 in neutrophils, suggesting that MA may confer anti-inflammatory benefits (2,3). MA is biosynthesized from oleic acid (18:1n-9; OA) through 6,9-octadecadienoic acid (18:2n-9; n-9 LnA) and 8,11-eicosadienoic acid (20:2n-9). A Δ12-desaturase-defective mutant of an arachidonic acid (20:4n-6)-producing fungus, *Mortierella alpina*, is a good producer of MA (4,5). The single-cell oil produced by the mutant strain contains *ca*. 69% n-9 FA (35% OA, 14% n-9 LnA, 3% 20:2n-9, and 17% MA) and 22% saturated FA. The activity of lipases on n-9 PUFA has not been reported, and the single-cell oil is a suitable substrate for study of this activity. In addition, n-9 LnA-rich and MA-rich preparations are valuable as chemical reagents for study of their physiological activities.

Lipase-catalyzed reactions are effective for purification of oil- and fat-related compounds (6). If components other than the desired one are converted to different molecular forms by selective reaction with an lipase, relatively easy purification can be obtained by combining with physical techniques, e.g., distillation, solvent fractionation, and urea adduct fractionation. Through processes composed of the selective reactions and physical procedures, several PUFA, such as GLA (18:3n-6), arachidonic acid, and docosahexaenoic acid (22:6n-3), have been enriched efficiently (6,7); isomers of CLA have also been fractionated and enriched (8–10). Hence, enzymatic fractionation and enrichment of n-9 LnA and MA from *M. alpina* single-cell oil was attempted. This paper shows that n-9 LnA-rich and MA-rich FFA can be prepared by selective hydrolysis with *Pseudomonas aeruginosa* lipase and selective esterification with *Candida rugosa* lipase.

MATERIALS AND METHODS

Materials. Single-cell oil (SUNTGM17) containing 17 wt% MA from *M. alpina* was a commercial product from Suntory Ltd. (Osaka, Japan). The saponification value was 184 mg KOH/g.

Fusarium heterosporum lipase was prepared as reported previously (11). In brief, the strain was aerobically cultivated at 27°C for 65 h in a medium (pH 5.5) containing 4% corn steep liquor, 3% soybean oil, 0.1% yeast extract, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7H_2O$. Ammonium sulfate was added to the culture filtrate to give 80% saturation, and the resulting precipitate was dialyzed against water. *Geotrichum candidum* lipase was prepared according to Tsujisaka *et al*. (12). The cells were cultivated at 27°C for 24 h in a medium (pH 6.0) containing 5% corn steep liquor, 1% soybean oil, and 0.5% NH_4NO_3. The enzyme solution was prepared by ammonium sulfate fractionation, followed by dialysis against water. The other lipases were obtained from the following companies: *P. aeruginosa* lipase (LPL; Toyobo Co. Ltd., Osaka, Japan); *Pseudomonas* sp. lipase (Lipase PS; Amano Enzyme Inc.,

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Aichi, Japan); *Pseudomonas* sp. lipase (Lipase AK; Amano Enzyme); *Burkholderia cepacia* lipase (Lipase SL; Meito Sangyo Co. Ltd., Aichi, Japan); *Alcaligenes* sp. lipase (Lipase QLM; Meito Sangyo); *C. rugosa* lipase (Lipase OF; Meito Sangyo); *Rhizopus oryzae* lipase (Ta-lipase; Tanabe Seiyaku Co., Osaka, Japan); *Rhizopus* sp. lipase (Lipase UL; Meito Sangyo); *Aspergillus niger* lipase (Lipase AP; Amano Enzyme).

Lauryl alcohol (LauOH) was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Other chemicals were of reagent grade.

Enzyme reactions. Reactions were conducted at 30°C in a 50-mL screw-capped vessel containing 4–10 g reaction mixture with stirring at 500 rpm. Hydrolysis of oils was performed at 30°C in a mixture containing 50% water and lipase. To study lipase activity on n-9 FA, a 6-g mixture of TGM17 oil/water (1:1, w/w) and 600 units (U) lipase was incubated at 30°C for 16 h with stirring, and the FA composition in the FFA fraction was analyzed. The degree of hydrolysis was determined by the acid value of the reaction mixture and the saponification value of the oil. Esterification of FFA was conducted in a mixture containing an equimolar amount of LauOH, 20% water, and lipase. The degree of esterification was calculated on the basis of the amount of FA consumed during the reaction.

Preparation of FFA from acylglycerols. Acylglycerols were chemically hydrolyzed under nitrogen gas in a 500-mL screw-capped vessel. A mixture of 100 g acylglycerols, 24 g NaOH, 30 mL water, and 300 mL ethanol was heated at 60°C for 60 min with stirring. After the reaction, 300 mL of 3 N HCl was added, and FFA were extracted two times with 200 mL *n*-hexane, which was finally removed by evaporation.

Fractionation of FFA and acylglycerols (lauryl esters) in the reaction mixture. After reaction, the hydrolysis reaction mixture contained FFA and acylglycerols, and the esterification mixture contained LauOH, FFA, and lauryl esters. FFA from the reaction mixtures were recovered by *n*-hexane extraction, as described in a previous paper (13). In brief, 50 mL of 0.5 N KOH (20% ethanol solution) was added to the reaction mixture containing 4–10 g of oil layer, and acylglycerols, lauryl esters, and LauOH were then extracted twice with 100 mL *n*-hexane. FFA remaining in the water layer were extracted twice with 100 mL *n*-hexane after acidification with HCl. A larger-scale extraction was performed on a fivefold scale.

Urea adduct fractionation. FFA (50 g) were 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 of 0.2 N HCl was added to the resulting filtrate, and FFA were recovered with 400 mL *n*-hexane.

Analyses. Lipase activity was measured by titrating FA liberated from olive oil (Wako Pure Chemical) with 50 mM KOH as described previously (14). The reaction was carried out at 30°C for 30 min with stirring at 500 rpm. One unit of lipase was defined as the amount that liberated 1 μmol of FA per minute.

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 acid value. FFA were methylated in 3 mL of 5% HCl/methanol by heating at 70°C for 10 min, and FA in acylglycerols 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, PA). The column temperature was raised from 150 to 210°C at 2°C/min, and the temperatures of injector and detector (FID) were set at 250°C.

RESULTS AND DISCUSSION

Activities of various lipases on n-9 FA. Activities of lipases on n-9 PUFA have not been reported; thus, the activities of various lipases were first measured using TGM17 oil as a substrate. The activity on a FA ester was expressed according to the following formula:

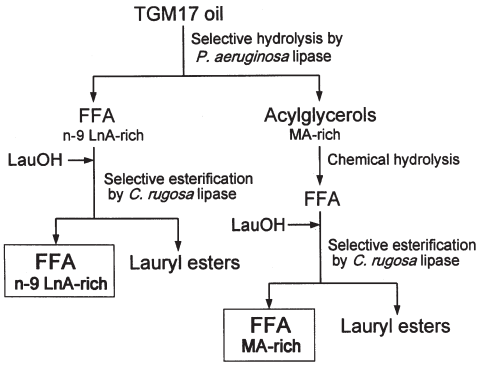
$$\text{activity} = F_{ffa}/F_{ori} \quad [1]$$

where F_{ffa} and F_{ori} are the contents (mol%) of a particular FA in the FFA fraction after the hydrolysis and in the substrate oil, respectively. Table 1 shows the degree of hydrolysis of TGM17 oil by each lipase and its relative activities against n-9 LnA and MA, which are expressed relative to the activity against OA in the oil. Lipases could be classified into three groups based on their activities against n-9 LnA and MA. The first group of lipases acted on n-9 LnA as strongly as on OA and acted weakly on MA. Among enzymes tested, only *P. aeruginosa* lipase belonged to this group. The second group acted weakly on both n-9 LnA and MA, and included *C. rugosa* and *G. candidum* lipases. The remaining lipases tested (Lipase QLM, Lipase PS, Lipase AK, Lipase SL, Ta-lipase, Lipase UL, *F. heterosporum*

TABLE 1
Activities of Several Lipases on n-9 FA[a]

Lipase	Hydrolysis (%)	Relative activity (%)[b]	
		n-9 LnA	MA
Pseudomonas aeruginosa	47.1	118	55
Candida rugosa	49.5	41	29
Geotrichum candidum	39.1	31	20
Alcaligenes sp.[c]	52.8	108	86
Pseudomonas sp.[d]	54.3	102	88
Pseudomonas sp.[e]	38.4	119	89
Burkholderia cepacia	53.5	110	80
Rhizopus oryzae	34.6	126	129
Rhizopus sp.[f]	36.4	119	123
Fusarium heterosporum	41.8	113	121
Aspergillus niger	52.6	85	92

[a]A mixture of 3 g TGM17 oil (Suntory Ltd., Osaka, Japan), 3 g water, and 100 U/g-reaction mixture lipase was incubated at 30°C for 16 h with stirring. The contents of oleic acid (OA), n-9 linoleic acid (LnA), and Mead acid (MA) in TGM17 oil were 36.4, 14.8, and 16.2 mol%, respectively.
[b]Activities on n-9 LnA and MA were expressed relative to that on OA.
[c]Lipase QLM.
[d]Lipase PS.
[e]Lipase AK.
[f]Lipase UL.



SCHEME 1

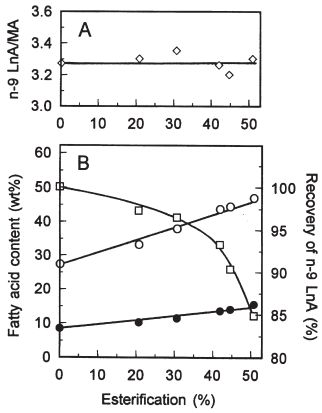


FIG. 1. Enrichment of n-9 linoleic acid (n-9 LnA) by selective esterification of FFA rich in n-9 LnA with lauryl alcohol (LauOH) using *Candida rugosa* lipase. A 4-g mixture of FFA/LauOH (1:1, mol/mol), 20% water, and 70, 100, 200, 400, and 700 U/g-reaction mixture of the lipase was stirred at 30°C for 16 h. FA composition in the FFA fraction was analyzed. (A) The ratio of n-9 LnA/Mead acid (MA); (B) the contents of n-9 LnA and MA, and recovery of n-9 LnA. ○, n-9 LnA content; ●, MA content; □, recovery of n-9 LnA; ◇, ratio of n-9 LnA/MA.

lipase, and *A. niger* lipase) belonged to the third group, which acted on n-9 LnA and MA as strongly as on the other constituent FA. Unfortunately, we did not find a lipase that acts strongly on MA and weakly on n-9 LnA.

A strategy for fractionation and enrichment of n-9 LnA and MA. The strategy is shown in Scheme 1. The first step is fractionation of n-9 LnA and MA. Hydrolysis of TGM17 oil with *P. aeruginosa* lipase liberates n-9 LnA from TGM17 oil but leaves MA in acylglycerols; thus, n-9 LnA and MA can be fractionated roughly into the FFA and acylglycerols, respectively. The second step is enrichment of the two FA by selective esterification, in which LauOH is a superior substrate (6,7). As *C. rugosa* lipase acted weakly on the two FA, selective esterification of the FFA obtained by the first-step hydrolysis with LauOH using the lipase can enrich n-9 LnA in the FFA fraction by removing other FFA as their lauryl esters. On the other hand, MA can be enriched by similar selective esterification of FFA originating from the acylglycerols that were obtained by the first-step hydrolysis. We attempted fractionation and enrichment of n-9 LnA and MA according to this strategy.

First step: fractionation of n-9 LnA and MA. TGM17 oil was hydrolyzed at 30°C for 16 h in a mixture containing 50% water and different amounts of *P. aeruginosa* lipase (Table 2). The degree of hydrolysis increased when larger amounts of the lipase were used. n-9 LnA content in the FFA fraction decreased with increasing hydrolysis, and the MA content in the acylglycerol fraction increased. If a higher concentration of n-9 LnA is desired, the hydrolysis should be kept to a low degree. However, because the low degree of hydrolysis produces smaller amounts of FFA, the amount of lipase was fixed at 50 U/g-reaction mixture in this study.

Second step: enrichment of n-9 LnA. *Candida rugosa* lipase has been reported to be a suitable catalyst for enrichment of arachidonic acid (15) and for fractionation and enrichment of CLA isomers (10). The selective esterification proceeded most efficiently when equimolar amounts of LauOH and FFA were used in a mixture containing 20% water. The content and recovery of a desired FA depended on the degree of esterification alone under these conditions. The reaction conditions for esterification of FFA originating from TGM17 oil were therefore set as follows: molar ratio of FFA/LauOH, 1:1; water content, 20%; temperature, 30°C.

The relationship between the degree of esterification and n-9 LnA content in FFA was investigated using FFA obtained from the first-step hydrolysis of TGM17 oil with *P. aeruginosa*

TABLE 2
Selective Hydrolysis of TGM17 Oil with *Pseudomonas aeruginosa* Lipase[a]

Enzyme amount (U/g-reaction mixture)	Hydrolysis (%)	Fraction	FA composition (wt%) OA	n-9 LnA	MA
0	0	Acylglycerol	35.4	14.3	17.1
10	24.5	Acylglycerol	34.8	11.5	20.9
		FFA	38.1	23.1	5.4
50	33.2	Acylglycerol	33.9	11.2	22.5
		FFA	38.4	20.5	6.2
100	46.9	Acylglycerol	33.7	11.7	23.0
		FFA	37.3	17.3	10.2

[a]A mixture of 3 g TGM17 oil (Suntory Ltd., Osaka, Japan), 3 g water, and 10, 50, and 100 U/g-reaction mixture of *P. aeruginosa* lipase (Toyobo Co. Ltd., Osaka, Japan) was incubated at 30°C for 16 h with stirring. For abbreviations see Table 1.

TABLE 3
Enrichment of n-9 LnA Recovered in FFA Fraction of Hydrolyzed TGM17 Oil

Step	Weight (g)	FA composition (wt%) OA	n-9 LnA	MA	n-9 LnA recovery (%)
TGM17 oil	250	35.4	14.3	17.1	100
Selective hydrolysis[a]					
FFA fraction	88.1	37.9	20.4	6.3	50.3
Urea adduct	62.8	45.4	27.2	8.3	47.8
Selective esterification[b]					
FFA fraction	34.3	18.1	46.8	15.4	44.9
Selective esterification[b]					
FFA fraction	25.3	9.8	54.0	19.0	38.2

[a]Selective hydrolysis of TGM17 oil was conducted at 30°C for 16 h in a mixture of 50% water and 50 U/g-reaction mixture *P. aeruginosa* lipase. For manufacturers see Table 2.
[b]Selective esterification of FFA was conducted at 30°C for 16 h with an equimolar amount of lauryl alcohol and FFA using 300 U/g-reaction mixture *C. rugosa* lipase (Meito Sangyo, Aichi, Japan). For abbreviations see Table 1.

lipase (Fig. 1). The degree of esterification was controlled by the amount of lipase. The increase in contents of n-9 LnA and MA depended on the degree of esterification, and the ratio of n-9 LnA/MA in the FFA fraction was constant at <50% esterification. Because >45% esterification decreased the recovery of n-9 LnA, the amount of lipase and reaction time were fixed at 300 U/g-reaction mixture and 16 h, respectively.

Fractionation and enrichment of n-9 LnA. Table 3 summarizes the enrichment of n-9 LnA from TGM17 oil. The first-step hydrolysis was conducted at 30°C for 16 h in a 50-mL reaction vessel containing 10 g of the oil/water (1:1, w/w) and 50 U/g-reaction mixture *P. aeruginosa* lipase. For the hydrolysis, 50 vessels were used in total, and the degree of hydrolysis reached 33.2% on average. The reaction mixture was separated by *n*-hexane extraction to yield 88.1 g FFA (n-9 LnA content, 20.4 wt%) and 149.5 g acylglycerols. The FFA were used for enrichment of n-9 LnA, and the acylglycerols were used for the enrichment of MA as described in the next section. Because the FFA fractions were in the solid state at the reaction temperature (30°C), saturated FA were removed by urea adduct fractionation. n-9 LnA content in the resulting FFA increased to 27.2 wt%. A mixture of the FFA/LauOH (1:1, mol/mol), 20% water, and 300 U/g-reaction mixture *C. rugosa* lipase was stirred at 30°C for 16 h. The degree of esterification reached 42.7%, and n-9 LnA content in FFA increased to 46.8 wt%. To further increase the n-9 LnA content, FFA were recovered by *n*-hexane extraction and were allowed to react again under the same conditions. The degree of esterification reached 21.9%, and 25.3 g FFA was recovered. This series of procedures increased n-9 LnA content to 54.0 wt% with a 38.2% recovery of the initial content in TGM17 oil.

Fractionation and enrichment of MA in FFA. The acylglycerol fraction (149.5 g; MA content, 23.7 wt%) obtained by hydrolysis of TGM17 oil with *P. aeruginosa* lipase was used for enrichment of MA (Table 4). The acylglycerols were first hydrolyzed chemically under alkaline conditions, and 133.8 g FFA was obtained. As the FFA mixture included saturated FA and was in the solid state at 30°C, urea adduct fractionation was performed to obtain the liquid-state FFA (MA content, 31.5 wt%). Selective esterification of the FFA with LauOH was

TABLE 4
Enrichment of MA Recovered in Acylglycerol Fraction of Hydrolyzed TGM17 Oil

Step	Weight (g)	FA composition (wt%) OA	n-9 LnA	MA	n-9 LnA recovery (%)
TGM17 oil	250	35.4	14.3	17.1	100
Selective hydrolysis[a]					
Acylglycerol fraction[a]	149.5	33.1	10.7	23.7	82.9
Chemical hydrolysis					
FFA fraction	133.8	33.0	11.1	24.4	76.4
Urea adduct	98.3	37.6	14.2	31.5	72.4
Selective esterification[a]					
FFA fraction	49.8	5.9	20.8	54.7	63.7
Selective esterification[b]					
FFA fraction	38.0	1.2	20.1	60.2	53.5

[a]Acylglycerols were derived from selective hydrolysis of TGM17 oil conducted as shown in Table 3.
[b]Reaction conditions were the same as those described in Table 3. For manufacturer and abbreviations see Table 1.

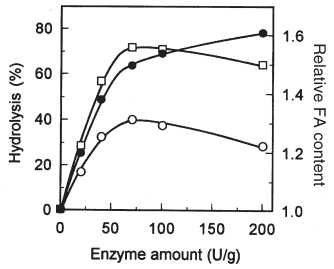


FIG. 2. Enrichment of MA in acylglycerol fraction by selective hydrolysis. The substrate was the acylglycerol fraction obtained by selective hydrolysis of TGM17 oil with *P. aeruginosa* lipase (Table 3). The degree of hydrolysis was 31.7%, and the resulting acylglycerols contained 23.0 wt% MA and 10.2 wt% n-9 LnA. A 4-g mixture of the acylglycerols/water (1:1, w/w) and 20, 40, 70, 100, and 200 U/g-reaction mixture of *C. rugosa* lipase was incubated at 30°C for 16 h. The contents of n-9 LnA and MA in the acylglycerol fraction were expressed relative to the substrate. ●, Degree of hydrolysis ; ○, n-9 LnA content; □, MA content.

n-9 LnA, owing to the fact that the lipase acted on n-9 LnA more strongly than on MA (Table 1). When the MA-rich oil was hydrolyzed with 70 U/g-reaction mixture of the lipase, the MA content reached a maximal value (36.2 wt%) with 57% recovery. n-9 LnA content in the acylglycerols was 13.5 wt%.

Comparison of selective esterification and hydrolysis. In this study, we obtained n-9 LnA-rich FFA, MA-rich FFA, and MA-rich acylglycerols. FA compositions of the preparations are shown in Table 5. Because we did not perform urea adduct fractionation for preparation of MA-rich acylglycerols, the acylglycerols contained 17.5% saturated FA in total. Even though all of the saturated FA were eliminated, MA content would increase to only 44.7 wt% [= 36.9/(1 – 0.175)]. The content was lower than that in the FFA (54.7 wt%) obtained by the single selective esterification for enrichment of MA, showing that FA selectivity of *C. rugosa* lipase was more strict in the esterification with LauOH than in the hydrolysis. In conclusion, the process composed of selective hydrolysis with *P. aeruginosa* lipase and selective esterification with *C. rugosa* lipase was more effective for fractionation and enrichment of n-9 LnA and MA than the process composed of two selective hydrolyses with *P. aeruginosa* and *C. rugosa* lipases.

TABLE 5
FA Compositions of n-9 LnA- and MA-rich Preparations Obtained in This Study

	FA composition (wt%)								
Preparation	16:0	18:0	18:1	18:2	20:1	20:2	20:3	22:0	24:0
TGM17 oil	7.8	5.6	35.4	14.3	2.2	2.7	17.1	2.4	5.9
n-9 LnA-rich FFA[a]									
Single reaction	4.5	1.4	18.1	46.8	3.1	5.1	15.4	ND[b]	ND
Repeated reaction	2.0	0.7	9.8	54.0	3.1	6.0	19.9	ND	ND
MA-rich FFA[c]									
Single reaction	0.3	0.4	5.9	20.8	3.9	6.4	54.7	ND	ND
Repeated reaction	ND	ND	1.2	20.1	3.9	6.6	60.2	ND	ND
MA-rich acylglycerols[d]	2.8	2.6	17.7	14.2	2.4	3.9	36.9	3.5	8.6

[a]FFA obtained through the process described in Table 3.
[b]Not detected.
[c]FFA obtained through the process described in Table 4.
[d]Acylglycerols obtained by selective hydrolysis with 70 U/g-reaction mixture *C. rugosa* lipase, as described in Figure 2.

conducted under the same conditions as those in the reaction for enrichment of n-9 LnA. The degree of esterification reached 46.6%. The MA content in the FFA fraction increased from 31.5 to 54.7 wt%, although n-9 LnA content also increased from 14.2 to 20.8 wt%. To increase the MA content further, selective esterification was repeated under the same conditions, resulting in a degree of esterification of 14.9%. Through this series of processes, 38.0 g FFA was recovered, in which MA and n-9 LnA contents were 60.2 and 20.1 wt%, respectively. The recovery of MA was 53.5% of the initial content in TGM17 oil.

Enrichment of MA in acylglycerols. As described in Tables 3 and 4, selective hydrolysis of TGM17 oil with *P. aeruginosa* lipase produced MA-rich acylglycerols (MA content, 23.7 wt%). To enrich MA further in the acylglycerol fraction, the MA-rich acylglycerol fraction was hydrolyzed with *C. rugosa* lipase. The water amount and reaction time were fixed at 50% and at 16 h, respectively, and the degree of hydrolysis was controlled by the amount of lipase. Figure 2 shows the relationship between the degree of hydrolysis and MA content in acylglycerols. The degree of hydrolysis increased as the amount of lipase was increased. MA was enriched more efficiently than

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