Production of Structured Lipids Containing Essential Fatty Acids by Immobilized *Rhizopus delemar* Lipase

Yuji Shimada[a,*], Akio Sugihara[a], Hirofumi Nakano[a], Tomomi Yokota[b], Toshihiro Nagao[a], Sadao Komemushi[b], and Yoshio Tominaga[a]

[a]Osaka Municipal Technical Research Institute, Joto-ku, Osaka 536, Japan, and
[b]Department of Agricultural Chemistry, School of Agriculture, Kinki University, Nakamachi, Nara 631, Japan

ABSTRACT: An attempt was made to produce structured lipids containing essential fatty acid by acidolysis with 1,3-positional specific *Rhizopus delemar* lipase. The lipase was immobilized on a ceramic carrier by coprecipitation with acetone and then was activated by shaking for 2 d at 30°C in a mixture of 5 g safflower or linseed oil, 10 g caprylic acid, 0.3 g water and 0.6 g of the immobilized enzyme. The activated enzyme was transferred into the same amount of oil/caprylic acid mixture without water, and the mixture was shaken under the same conditions as for the activation. By this reaction, 45–50 mol% of the fatty acids in oils were exchanged for caprylic acid, and the immobilized enzyme could be reused 45 and 55 times for safflower and linseed oils, respectively, without any significant loss of activity. The triglycerides were extracted with *n*-hexane after the acidolysis and then were allowed to react again with caprylic acid under the same conditions as mentioned above. When acidolysis was repeated three times with safflower oil as a starting material, the only products obtained were 1,3-capryloyl-2-linoleoylglycerol and 1,3-capryloyl-2-oleoyl-glycerol, with a ratio of 86:14 (w/w). Equally, the products from linseed oil were 1,3-capryloyl-2-α-linolenoyl-glycerol, 1,3-capryloyl-2-linoleoyl-glycerol, and 1,3-capryloyl-2-oleoyl-glycerol (60:22:18, w/w/w). All fatty acids at the 1,3-positions in the original oils were exchanged for caprylic acid by the repeated acidolyses, and the positional specificity of *Rhizopus* lipase was also confirmed to be strict.
JAOCS 73, 1415–1420 (1996).

KEY WORDS: Acidolysis, caprylic acid, essential fatty acid, immobilized enzyme, linseed oil, *Rhizopus delemar* lipase, safflower oil, structured lipid.

Lipase reactions proceed under ordinary temperature and pressure and are able to take advantage of specificities. In addition, not only hydrolysis but also esterification and transesterification can be conducted by controlling water content in the reaction system (1,2). These lipase-catalyzed reactions have been applied to high-level processing of oils and fats, and many studies have been reported. Polyunsaturated fatty acids (PUFA) with various physiological functions are unstable to oxidation and heating, and lipases have little reactivity toward these fatty acids (3,4). Thus, docosahexaenoic acid (DHA), arachidonic acid (AA) and γ-linolenic acid (GLA) were enriched in glycerides by hydrolyses of tuna oil (5–7), singlecell oil from *Mortierella* (4), and borage oil (8), respectively. When fatty acids containing GLA or DHA were esterified with butanol by using lipases, these PUFA were enriched in the unesterified fatty acid fraction (9,10). In addition, cocoa butter-like fat was produced by acidolysis of olive oil with palmitic acid (PA) by using a 1,3-specific lipase (11). The DHA-rich oil was produced by acidolysis of fish oil with DHA-rich fatty acids (12,13). Furthermore, it has been reported that partial glycerides were produced by the alcoholysis of triglyceride with glycerol (14,15).

Pancreatic lipase hydrolyzes ester bonds at the 1- and 3-positions in triglycerides and shows higher activity toward medium-chain than toward long-chain fatty acids (3,16). Long-chain triglycerides (most of natural oils and fats) are hydrolyzed to 2-monoglycerides and fatty acids by the lipase, and the hydrolysis products are absorbed into the intestinal mucosa. Thus, triglycerides with medium-chain fatty acids at the 1- and 3-positions and with functional fatty acids at the 2-position are rapidly hydrolyzed with pancreatic lipase and absorbed efficiently into mucosal cells (17,18). These highly absorptive triglycerides are referred to as structured lipids, and are expected to be used as medicines, and in clinical nutrition and health foods. Akoh *et al.* reported a production method of the structured lipid that contains oleic acid (OA) and caprylic acid (CA) by immobilized *Rhizomucor miehei* lipase (19), and we also produced one containing DHA and CA with immobilized *Rhizopus delemar* lipase (20). On the other hand, structured lipids with essential fatty acids are available for the care of preterm infants and patients with maldigestion and malabsorption, and they are also of interest as nutrition for other groups of patients (21,22).

Here, we describe an effective method of producing structured lipids that contain linoleic acid (LA) and α-linolenic acid (ALA) by acidolyses of safflower and linseed oils with CA, respectively, by means of immobilized *Rhizopus delemar* lipase.

MATERIALS AND METHODS

Oils and fatty acid. Safflower and linseed oils were purchased from Yamakei Sangyo Co. (Osaka, Japan). Caprylic acid was purchased from Wako Pure Chemical (Osaka, Japan).

*To whom correspondence should be addressed at Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536, Japan.

Preparation of immobilized lipase and acidolysis. Rhizopus delemar lipase (Ta-lipase, 120,000 U/g) was a gift from Tanabe Seiyaku Co. (Osaka, Japan). The lipase was immobilized on a ceramic carrier, SM-10, a gift from NGK Insulators (Aichi, Japan), as described in our previous paper (20). After the ceramic carrier (50 g) was suspended in 200 mL of 10% lipase solution, cold acetone (−80°C) was gradually added with stirring, and the precipitate was dried *in vacuo*. Approximately 90% of lipase was immobilized on the carrier by this procedure. To activate this immobilized enzyme, the following pretreatment was conducted: The mixture of 5 g safflower or linseed oil, 10 g CA, 0.3 g deionized water and 0.6 g immobilized enzyme was incubated in a screw-capped vessel (20 mL) at 30°C for 2 d with shaking at 140 oscillations/min. The activated enzyme was transferred into the same amount of the safflower or linseed oil/CA mixture without water, and then the acidolysis was conducted under the same conditions as those for the pretreatment. When the reaction was scaled up, the amounts of the components were increased with the same ratios.

Analysis. Lipase activity was measured by titrating fatty acids from olive oil (Wako Pure Chemical) with 0.05 N KOH, as described previously (23). The reaction was conducted at 30°C for 60 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount of the enzyme that liberated 1 μmol of fatty acid/min.

Glycerides were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) to 5 g of the reaction mixture. Fatty acids in glycerides were methylated in methanol with sodium methylate as a methylating reagent. These methyl esters were analyzed by a Hewlett-Packard 5890 *Plus* gas chromatograph (Avondale, PA) connected to a DB-23 capillary column (0.25 mm × 30 m, J&W Scientific, Folsom, CA) as described previously (5). The contents of mono-, di- and triglycerides were measured by a thin-layer chromatograph/flame-ionization detector analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol). Triglycerides were analyzed on two octadecyl silica (ODS) columns (4.6 × 150 mm, Wakosil-II3C18 HG, Wako Pure Chemical), connected with a high-performance liquid chromatography (HPLC) system (LC-9A; Shimadzu Co., Kyoto, Japan). The sample was eluted with a mixture of acetone/acetonitrile (1:1, vol/vol) at a flow rate of 0.4 mL/min and 40°C, and detected with a refractometer.

Fatty acid composition of 1(3)- and 2-positions in triglyceride. Regiospecific analysis of triglycerides was conducted by Grignard degradation with allyl magnesium bromide (24), followed by isolation and analysis of the 1,3-diglyceride fraction. The 1,3-diglycerides were isolated by thin-layer chromatography, which was developed with a mixture of chloroform/acetone/acetic acid (96:4:1, vol/vol/vol) (25). Fatty acid composition of the 2-position in the triglycerides $F(2)$ was calculated according to the following formula:

$$F(2) = F(\text{total}) - 2/3 \times F(1,3) \quad [1]$$

where $F(\text{total})$ and $F(1,3)$ are the fatty acid compositions (mol%) of undigested triglycerides and of 1,3-diglycerides obtained by Grignard degradation, respectively.

RESULTS

Activation of immobilized lipase. When immobilized *Rhizopus* lipase was pretreated as outlined in the Materials and Methods section, CA was incorporated at 43 and 44 mol% into the glycerides obtained from safflower and linseed oils, respectively (Table 1). Acidolysis occurred effectively in the pretreatment, but partial glycerides were generated, showing that hydrolysis occurred simultaneously. The activated-immobilized enzyme was transferred into an oil/CA mixture without water, and then acidolysis was carried out. As shown in Table 1, the simultaneous hydrolysis was repressed, and acidolysis was enhanced a little.

Time course of acidolysis. Acidolysis of safflower or linseed oil with CA was conducted by the immobilized lipase, which had been used twice. Figure 1 shows the change of fatty acid composition of the glyceride fraction. When safflower oil was used, the contents of LA, OA, and PA were decreased and that of CA was increased. The reaction reached a steady state after approximately 15 h, and 47 mol% CA was incorporated into the glycerides after a 50-h reaction. Similar results were obtained when linseed oil was used; 48 mol% CA was incorporated after 50 h of reaction. From these results, the reaction time was determined to be 2 d.

The increase in CA content was almost equivalent to the total decrease in fatty acids in safflower or linseed oil. This result confirmed that fatty acids in the oil were exchanged for CA and that hydrolysis scarcely occurred.

Fatty acid compositions of 1(3)- and 2-positions in transesterified oil. Table 2 shows the fatty acid compositions of the 1(3)- and 2-positions in the original oils and in the triglycerides obtained after their acidolyses with CA, which are named transesterified oils. Fatty acid compositions of the 2-position in the oils before and after the reaction were the same, and CA was not incorporated into the 2-position. This result showed that only fatty acids at the 1(3)-positions in saf-

TABLE 1
Pretreatment of Immobilized Lipase and Subsequent Acidolysis

Oil	Treatment	Incorporation of caprylic acid (mol%)	Glyceride content (wt%) TG[a]	DG[b]	MG[c]
Safflower	First[d]	43.3	72	26	2
	Second	46.6	92	8	ND[e]
	Third	45.7	99	1	ND
Linseed	First[d]	44.3	73	25	2
	Second	49.6	89	10	1
	Third	48.4	98	2	ND

[a]Triglycerides.
[b]Diglycerides.
[c]Monoglycerides.
[d]Pretreatment.
[e]Not detectable.

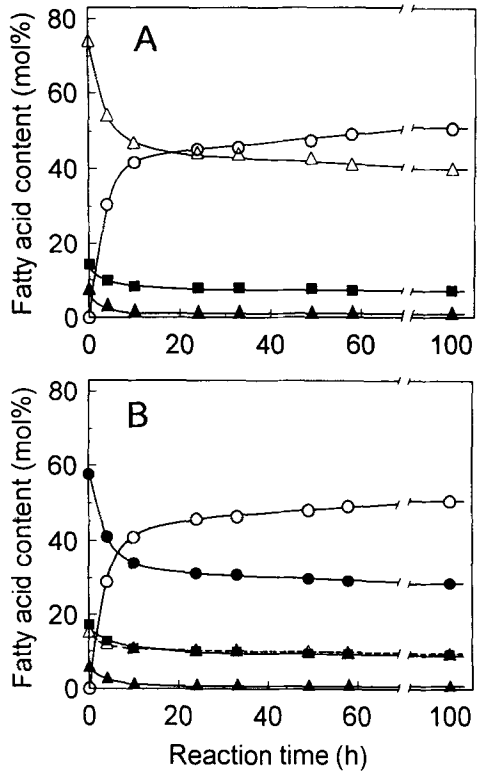

FIG. 1. Change of fatty acid composition in triglycerides obtained by acidolysis of (A) safflower oil and (B) linseed oil with caprylic acid. ○, Caprylic acid; ●, α-linolenic acid; △, linoleic acid; ■, oleic acid; ▲, palmitic acid.

flower and linseed oils were exchanged for CA by *Rhizopus* lipase.

Stability of immobilized lipase. Acidolysis was continuously performed by transferring the immobilized enzyme into a fresh oil/CA mixture every 2 d (Fig. 2). In the reactions with safflower and linseed oils, the enzyme could be reused 45 and 55 times, respectively, without any significant loss of activity, and 45–50 mol% of fatty acids in oils were exchanged for CA.

When the 56th reaction of safflower oil and the 63rd reaction of linseed oil were extended to 3 d, the CA incorporations recovered to 47 and 48%, respectively. After these reactions, 50% water relative to the amount of immobilized enzyme was added to the reaction mixtures, and the CA incorporation in subsequent reactions was investigated. The acidolysis activity did not recover, suggesting that the decline in activity was due to inactivation of the lipase or its release from the ceramic carrier, and not due to the release of water bound to immobilized enzyme.

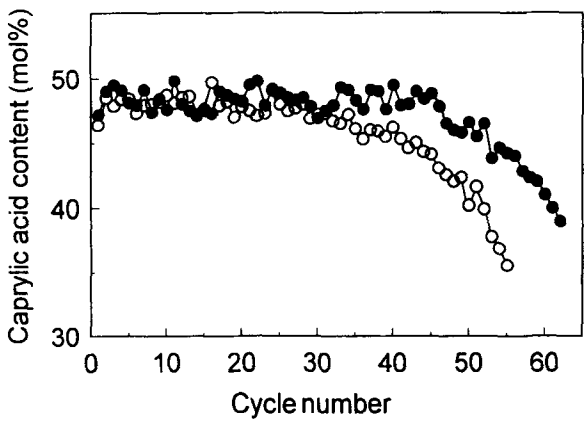

FIG. 2. Stability of immobilized lipase. The reaction was conducted as described in Materials and Methods. Acidolysis activity was expressed as the caprylic acid content incorporated into triglycerides. ○, Acidolysis of safflower oil; ●, acidolysis of linseed oil.

Increase in acidolysis extent by repeated reactions. The first acidolyses of safflower and linseed oils exchanged 73.3 and 69.9% of fatty acids at their 1(3)-positions for CA, respectively (Table 3). To elevate the extent of acidolysis, the glycerides extracted from the reaction mixtures were allowed to react again with CA. The content of fatty acids other than CA in the triglycerides, which were obtained after three reactions, agreed with the fatty acid content of the 2-position of safflower and linseed oils, respectively (Tables 2 and 3). These results suggested that fatty acids at the 1(3)-position in triglycerides were completely exchanged for CA by the repeated reactions.

Triglyceride components in transesterified oils. Triglycerides in transesterified oils were analyzed by HPLC. All triglycerides obtained by the first acidolyses of safflower and linseed oils were new components, which were eluted after

TABLE 2
Fatty Acid Compositions of 1(3)- and 2-Positions in Safflower and Linseed Oils, and Their Transesterified Oils

	Fatty acid content (mol%)							
	Safflower oil				Linseed oil			
Fatty	Original		Transesterified		Original		Transesterified	
acid	1(3)	2	1(3)	2	1(3)	2	1(3)	2
8:0	ND[a]	ND	49.7	ND	ND	ND	45.6	0.7
16:0	7.4	0.3	0.8	0.2	5.9	0.1	1.3	ND
18:0	2.3	0.2	0.5	ND	2.9	ND	0.7	ND
18:1	9.2	4.5	2.5	4.7	11.3	6.1	3.7	6.3
18:2	47.7	28.1	13.2	28.7	8.7	6.7	3.5	7.0
18:3	ND	ND	ND	ND	37.9	19.7	11.8	19.1

[a]Not detectable.

TABLE 3
Increase in Extent of Acidolysis by Repeated Reaction

Oil	Treatment	Fatty acid content (mol%) 8:0	16:0	18:0	18:1	18:2	18:3	Acidolysis extent[a] (%)
Safflower	None	ND[b]	7.7	2.5	13.5	74.3	ND	—
	First	48.9	1.4	0.5	7.2	41.9	ND	73.3
	Second	59.8	0.5	ND	5.7	34.0	ND	89.7
	Third	67.4	ND	ND	5.2	27.4	ND	101.2
Linseed	None	ND	6.0	2.9	16.7	15.4	57.6	—
	First	46.6	1.3	0.6	10.0	10.5	30.9	69.9
	Second	60.8	0.4	ND	7.6	8.4	22.7	91.2
	Third	66.7	ND	ND	6.3	7.3	19.7	100.0

[a]Expressed relative to fatty acids located at the 1(3)-position.
[b]Not detectable.

shorter retention times, compared with those of the triglycerides in the original oils (Fig. 3A, B, D, and E). Because tricaprylin eluted at 11.8 min, it showed that all transesterified oil contained one or two CA at the 1(3)-position.

To identify the triglyceride components fractionated by HPLC, the peaks numbered in Fig. 3B and E were collected, and their fatty acid compositions were analyzed (Table 4). On the basis of the molar ratio of fatty acids, the structures of peaks 1–5 in transesterified oil derived from safflower oil (Fig. 3B) were estimated as follows: peak 1, 1,3-capryloyl-2-linoleoyl-glycerol; peak 2, 1,3-capryloyl-2-oleoyl-glycerol; peak 3, triglyceride with one molecule of CA and two molecules of LA; peak 4, triglyceride with CA, LA, and OA; and peak 5, triglyceride with CA, LA, and PA. The structures of peaks 1–6 in transesterified oil produced from linseed oil (Fig. 3E) were estimated as follows: peak 1, 1,3-capryloyl-2-α-linolenoyl-glycerol; peak 2, 1,3-capryloyl-2-linoleoyl-glycerol; peak 3, triglyceride with one molecule of CA and two molecules of ALA; peak 4, 1,3-capryloyl-2-oleoyl-glycerol; peak 5, triglycerides with CA, ALA, and LA; peak 6, triglyceride with CA, ALA, and OA.

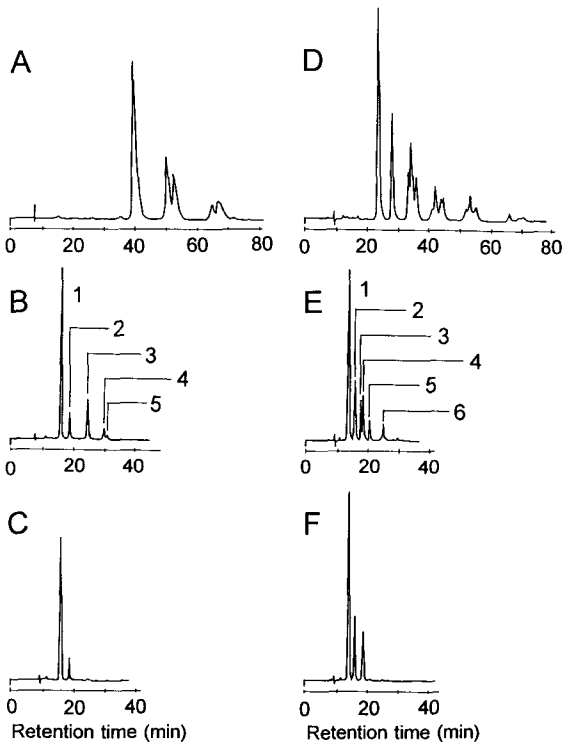

FIG. 3. High-performance liquid chromatography analyses of triglycerides obtained by repeated acidolyses of safflower and linseed oils. A, Safflower oil; B and C, triglycerides obtained by the first and third acidolyses of safflower oil, respectively; D, linseed oil; E and F, triglycerides obtained by the first and third acidolyses of linseed oil, respectively. The peaks numbered in B and E were collected, and their fatty acid compositions were analyzed.

When acidolysis was repeated three times with safflower oil as a starting material, only 1,3-capryloyl-2-linoleoyl-glycerol and 1,3-capryloyl-2-oleoyl-glycerol existed in the resulting triglycerides, and their contents, calculated from the peak areas, were 86 and 14%, respectively (Fig. 3C). Additionally, all triglycerides in linseed oil were converted to 1,3-capryloyl-2-α-linolenoyl-glycerol, 1,3-capryloyl-2-linoleoyl-glycerol, and 1,3-capryloyl-2-oleoylglycerol by repeating the reaction three times; their contents were 60, 22, and 18%, re-

TABLE 4
Fatty Acid Compositions of Triglycerides Fractionated by High-Performance Liquid Chromatography

Triglyceride[a]	Retention time (min)	Fatty acid composition (mol%) 8:0	16:0	18:1	18:2	18:3
Safflower oil						
Peak 1	16.1	64.0	ND[b]	ND	36.0	ND
Peak 2	19.0	62.5	0.7	35.3	1.6	ND
Peak 3	24.8	31.6	ND	0.4	68.0	ND
Peak 4	30.4	31.6	1.4	31.3	35.6	ND
Peak 5	31.5	31.8	30.4	3.2	34.5	ND
Linseed oil						
Peak 1	14.2	64.5	ND	1.0	0.2	34.2
Peak 2	16.2	65.3	ND	0.8	32.0	1.8
Peak 3	18.3	31.2	ND	3.7	0.4	64.7
Peak 4	19.0	60.4	0.8	35.7	0.4	2.6
Peak 5	21.2	32.3	0.9	1.4	32.5	32.9
Peak 6	25.5	27.9	2.7	27.3	7.6	34.5

[a]Peaks 1–5 (from safflower oil) and peaks 1–6 (from linseed oil) correspond to the components numbered in Figure 3B and E, respectively.
[b]Not detectable.

spectively (Fig. 3F). The contents of these structured lipids agreed with the fatty acid compositions of the 2-positions in safflower and linseed oils, respectively (Table 2). From these results, it was confirmed that all fatty acids at the 1- and 3-positions of triglycerides were exchanged for CA by repeated acidolyses. This shows that the positional specificity of *Rhizopus* lipase is extremely strict in acidolysis.

DISCUSSION

We have described an effective method of producing structured lipids that contain essential fatty acids by the exchange of fatty acids at the 1(3)-positions in safflower and linseed oils for CA by means of immobilized *Rhizopus delemar* lipase. It has also been shown that the repeated reaction was effective for the elevation of CA extent, and that fatty acids at the 1(3)-position of triglyceride were completely exchanged by repeating the process three times. Because the structured lipid containing DHA was produced by the same reaction system as described here (20), this system may also be available to produce other structured lipids, *e.g.*, triglycerides containing GLA and AA from borage oil and *Mortierella* single-cell oil, respectively.

Comparison of acidolyses of safflower and linseed oils with that of tuna oil. Immobilized *Rhizopus* lipase could be reused 45 times in the reaction of safflower oil and 55 times in that of linseed oil, without any significant loss of activity (Fig. 2). On the other hand, the enzyme could be reused only 15 times in acidolysis of tuna oil (20). The reactions of safflower and linseed oils reached steady states after 15 h (Fig. 1), but that of tuna oil required 40 h (20). These results suggest that the long life of immobilized enzyme in the reactions of safflower and linseed oils was attributed to the use of an excess amount of enzyme.

The period necessary for the reaction of tuna oil was longer than for the reactions of safflower and linseed oils. Safflower and linseed oils are composed of only fatty acids on which the lipase acts well, but tuna oil contains fatty acids on which the lipase acts poorly; especially DHA and eicosapentaenoic acid (EPA). Therefore, oils that are good substrates of the lipase may be transesterified at higher reaction rates.

Application of acidolysis with immobilized lipase to analytical methods. When lipase is used for oil processing as a catalyst, the fatty acid specificity becomes an important factor. However, lipases simultaneously catalyze hydrolysis, esterification, and transesterification under reaction conditions that include the presence of water (6,26), and it becomes difficult to evaluate the fatty acid specificity in each reaction. Because only acidolysis occurred in the reaction system described here, the preliminary evaluation of the fatty acid specificity of *Rhizopus* lipase in acidolysis was on the basis of the time courses of acidolyses of tuna (20), safflower, and linseed oils (Fig. 2). As a result, PA, palmitoleic acid, stearic acid, OA, LA, and ALA were well exchanged for CA at the same rate. EPA and AA were gradually exchanged, but DHA was scarcely exchanged. This fatty acid specificity in acidolysis was similar to that in hydrolysis, although DHA ester was lightly hydrolyzed (4). To confirm that acidolysis activity on DHA is extremely low, acidolysis of tuna oil with CA was repeated three times. As expected, DHA at the 1(3)-position in triglycerides was scarcely exchanged, although the other fatty acids were completely exchanged (data not shown). This result shows that the fatty acid specificity, evaluated in the reaction system in this study, can be applied to the prediction of acidolysis with the lipase.

Regiospecific analysis of triglyceride has been conducted by Grignard degradation (24). When acidolyses of safflower and linseed oils were repeated three times, all fatty acids at the 1(3)-position in triglyceride were exchanged for CA (Table 3 and Fig. 2), and the fatty acid compositions (except for CA) of the transesterified oils agreed with those of the 2-positions in the original oils (Tables 2 and 3). Therefore, this reaction can be used as a method of analyzing fatty acid composition of the 2-position in natural oil consisting of only fatty acids on which *Rhizopus* lipase acts well.

REFERENCES

1. Iwai, M., Y. Tsujisaka, and J. Fukumoto, Studies on Lipase. II. Hydrolytic and Esterifying Actions of Crystalline Lipase of *Aspergillus niger*, *J. Gen. Appl. Microbiol. 10*:13–22 (1964).
2. Yamane, T., Enzyme Technology for the Lipids Industry: An Engineering Overview, *J. Am. Oil Chem. Soc. 64*:1657–1660 (1987).
3. Yang, L.-Y., A. Kuksis, and J.J. Myher, Lipolysis of Menhaden Oil Triacylglycerols and the Corresponding Fatty Acid Alkyl Esters by Pancreatic Lipase *in vitro*: A Reexamination, *J. Lipid Res. 31*:137–148 (1990).
4. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, and Y. Tominaga, Enrichment of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with *Candida cylindracea* Lipase, *J. Am. Oil Chem. Soc. 72*:1323–1327 (1995).
5. Shimada, Y., K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase, *Ibid. 71*:951–954 (1994).
6. Shimada, Y., K. Maruyama, M. Nakamura, S. Nakayama, A. Sugihara, and Y. Tominaga, Selective Hydrolysis of Polyunsaturated Fatty Acid-Containing Oil with *Geotrichum candidum* Lipase, *Ibid. 72*:1577–1581 (1995).
7. Tanaka, Y., J. Hirano, and T. Funada, Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with *Candida cylindracea* Lipase, *Ibid. 69*:1210–1214 (1992).
8. Rahmatullar, M.S.K.S., V.K.S. Shukla, and K.D. Mukherjee, Enrichment of γ-Linolenic Acid from Evening Primrose Oil and Borage Oil *via* Lipase-Catalyzed Hydrolysis, *Ibid. 71*:569–573 (1994).
9. Foglia, T.A., and P.E. Sonnet, Fatty Acid Selectivity of Lipase: γ–Linolenic Acid from Borage Oil, *Ibid. 72*:417–420 (1994).
10. Rahmatullar, M.S.K.S., V.K.S. Shukla, and K.D. Mukherjee, γ-Linolenic Acid Concentrates from Borage and Evening Primrose Oil Fatty Acids *via* Lipase-Catalyzed Esterification, *Ibid. 71*:563–567 (1994).
11. Yokozeki, K., S. Yamanaka, K. Takinami, Y. Hirose, A. Tanaka, K. Sonomoto, and S. Fukui, Application of Immobilized Lipase to Regio-Specific Interesterification of Triglyceride

in Organic Solvent, *J. Appl. Microbiol. Biotechnol. 14*:1–5 (1982).
12. Osada, K., K. Takahashi, and M. Hatano, Modification of Sardine Oil by Immobilized Lipase, *Yukagaku 39*:467–471 (1990). (in Japanese)
13. Adachi, S., K. Okumura, Y. Ota, and M. Mankura, Acidolysis of Sardine Oil by Lipase to Concentrate Eicosapentaenoic and Docosahexaenoic Acids in Glycerides, *J. Ferment. Bioeng. 75*:259–264 (1993).
14. McNeill, G.P., and T. Yamane, Further Improvements in the Yield of Monoglycerides During Enzymatic Glycerolysis of Fats and Oils, *J. Am. Oil Chem. Soc. 68*:6–10 (1991).
15. Yamane, T., S.T. Kang, K. Kawahara, and Y. Koizumi, High-Yield Diacylglycerol Formation by Solid-Phase Enzymatic Glycerolysis of Hydrogenated Beef Tallow, *Ibid. 71*:339–342 (1994).
16. Bottino, N.R., G.A. Vandenburg, and R. Reiser, Resistance of Certain Long Chain Polyunsaturated Fatty Acids of Marine Oil to Pancreatic Lipase Hydrolysis, *Lipids 2*:489–493 (1967).
17. Christensen, M.S., C.-E. Hoy, C.C. Becker, and T.G. Redgrave, Intestinal Absorption and Lymphatic Transport of Eicosapentaenoic (EPA), Docosahexaenoic (DHA), and Decanoic Acids: Dependence on Intramolecular Triacylglycerol Structure, *Am. J. Clin. Nutr. 61*:56–61 (1995).
18. Ikeda, I., Y. Tomari, M. Sugano, S. Watanabe, and J. Nagata, Lymphatic Absorption of Structured Glycerolipids Containing Medium-Chain Fatty Acids and Linoleic Acid, and Their Effect on Cholesterol Absorption in Rats, *Lipids 26*:369–373 (1991).
19. Shieh, C.-J.S., C.C. Akoh, and P.E. Koehler, Four-Factor Response Surface Optimization of the Enzymatic Modification of Triolein to Structured Lipids, *J. Am. Oil Chem. Soc. 72*:619–623 (1995).
20. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, and Y. Tominaga, Production of Structured Lipid Containing Docosahexaenoic and Caprylic Acids Using Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 81*:299–303 (1996).
21. Babayan, V. K., Medium Chain Triglycerides and Structured Lipids, *Lipids 22*:417–420 (1987).
22. Björkling, F., S.E. Godtfredsen, and O. Kirk, The Future Impact of Industrial Lipases, *TIBTECH 9*:360–363 (1991).
23. Sugihara, A., Y. Shimada, and Y. Tominaga, Separation and Characterization of Two Molecular Forms of *Geotrichum candidum* Lipase, *J. Biochem. 107*:426–430 (1990).
24. Becker, C.C., A. Rosenquist, and G. Holmer, Regiospecific Analysis of Triacylglycerols Using Allyl Magnesium Bromide. *Lipids 28*:147–149 (1993).
25. Sugihara, A., M. Ueshima, Y. Shimada, S. Tsunasawa, and Y. Tominaga, Purification and Characterization of a Novel Thermostable Lipase From *Pseudomonas cepacia, J. Biochem. 112*:598–603 (1992).
26. Okumura, S., M. Iwai, and Y. Tsujisaka, The Effect of Reverse Action on Triglyceride Hydrolysis by Lipase, *Agric. Biol. Chem. 45*:185–189 (1981).

[Received July 8, 1996; accepted August 5, 1996]