Selective Hydrolysis of Polyunsaturated Fatty Acid-Containing Oil with *Geotrichum candidum* Lipase

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ABSTRACT: Polyunsaturated fatty acids (PUFA), especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), can be concentrated in glycerides by hydrolyzing tuna oil with *Geotrichum candidum* lipase, the main components in the resulting oil being triglycerides. The reaction mechanism of this selective hydrolysis was investigated. Although the lipase acted well on the esters of oleic, linoleic, and α-linolenic acids, it did not affect the esters of γ-linolenic acid, arachidonic acid, EPA, and DHA as much. The action of PUFA-glycerides was mono- > di- > triglycerides. Furthermore, the condensation of PUFA-partial glycerides and PUFA occurred even in the presence of a large amount of water, and the partial glycerides converted to the triglycerides by transacylation. These results suggested that the PUFA-rich triglycerides were accumulated in the glyceride fraction by the following mechanism: The PUFA-partial glycerides generated by the hydrolysis were converted to PUFA-triglycerides by condensation and transacylation reactions. As the PUFA-triglycerides formed were the poor substrates of lipase, they were accumulated in the reaction mixture.
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KEY WORDS: Enrichment, fatty acid specificity, *Geotrichum candidum* lipase, glyceride specificity, polyunsaturated fatty acids, selective hydrolysis.

Since epidemiologic studies were reported by Bang *et al.* (1) in 1978, n-3 polyunsaturated fatty acids (PUFA) have become potential pharmaceutical substances. Eicosapentaenoic acid (20:5, EPA) and docosahexaenoic acid (22:6, DHA) especially play a role in the prevention of a number of human diseases (2–7). These physiological functions drew attention to the subject, and PUFA-rich oil has been produced by the traditional method of winterization (8). However, the yield was very low, and about 35% was the limit value of the enrichment of DHA. Recently, it was shown that PUFA was concentrated in glycerides by the hydrolysis of tuna oil with lipase (9–11), and this selective hydrolysis became available for the production of PUFA-rich oil.

The lipases from *Geotrichum candidum* and *Candida rugosa* are useful for the production of the oil containing high concentration of PUFA (10,11). *Geotrichum candidum* lipase can enrich DHA and EPA (11), and *C. rugosa* lipase can enrich DHA, but not EPA (10,11). On the other hand, an oil containing a higher concentration of DHA can be achieved with the *C. rugosa* lipase than with the *G. candidum* enzyme, although the yield of DHA in the glyceride fraction is lower (11). The main components in the resulting oils were triglycerides (80–90%); monoglycerides are scarcely present (11). The enrichment of DHA and EPA with *G. candidum* lipase and of DHA with *C. rugosa* lipase depended on the hydrolysis extent of tuna oil with these lipases (11). But tuna oil is not hydrolyzed as much with these enzymes; its hydrolysis with *G. candidum* lipase was only *ca.* 30%, although the hydrolysis of olive oil was *ca.* 90%. It is very important to clarify the reaction mechanism causing these phenomena upon the application of lipase to oil processing. In this paper, we describe that the accumulation of PUFA-rich triglycerides by hydrolysis of tuna oil with *G. candidum* lipase can be explained by the fatty acid specificity and the activity on mono-, di-, and triglycerides of PUFA.

MATERIALS AND METHODS

Lipases. Geotrichum candidum ATCC34614 produces four kinds of lipases, I–IV, lipase I being a major component (12). The cultivation was carried out in a 30-L fermenter as described previously (13). Lipase I was used in this paper, and it was purified from 12 L of culture filtrate through four purification steps: ammonium sulfate fractionation; DEAE-Sephadex A-50 (Pharmacia LKB, Uppsala, Sweden) ion exchange chromatography; Sephacryl S-100 (Pharmacia LKB) gel filtration; and methyl-HIC (Bio-Rad Laboratories, Richmond, CA) hydrophobic interaction chromatography, as described previously (11). The lipases from *C. rugosa* (Lipase-OF) and *Chromobacterium viscosum* were gifts from Meito Sangyo Co. (Aichi, Japan) and Asahi Chemical Industry Co. (Tokyo, Japan), respectively.

Oils. Tuna oil, DHA30 (saponification value, 184; acid value, 0.05), refined by Maruha Co. (Tokyo, Japan) was used. Olive oil (saponification value, 195; acid value, 0.09) was purchased from Wako Pure Chemicals Inc. (Osaka, Japan).

Preparation of free fatty acids (FFA) from hydrolyzate of olive oil or tuna oil. A reaction mixture containing 4 g olive

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oil or tuna oil, 4 mL deionized water, and 800 units (U) *G. candidum* lipase was incubated at 30°C with stirring (500 rpm) for 16 h. Glycerides were removed by extracting twice with 100 mL *n*-hexane after adding 50 mL of 0.5 N ethanolic KOH to the hydrolysis reaction mixture. Fatty acids contained in the water phase were extracted two times with 100 mL *n*-hexane after adding 40 mL of 2 N HCl, and finally the organic solvent was evaporated. The fatty acids from the hydrolyzates of olive oil and tuna oil were 3.1 and 1.0 g, respectively.

Preparation of triglycerides containing different amount of PUFA. Tuna oil (3 g) was applied to a DEVELOSIL ODS column (50 × 500 mm; Nomura Chemical Co., Aichi, Japan) connected to a preparative HPLC system Model 100 (Mitsubishi Kakoki Kaisha Ltd., Tokyo, Japan), and separated into ten fractions by elution with a mixture of acetone/acetonitrile (7:2, vol/vol) at a flow rate of 60 mL/min. The elution was monitored with the absorbance at 214 nm. The resulting fractions were specified by applying to two columns (4.6 × 150 mm, Wakosil-II3C18 HG; Wako Pure Chemicals Inc.) in series connected to a Shimadzu LC-10AS HPLC system (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 refractive index.

Separation of di- and triglycerides from tuna oil treated with lipase. The reaction mixture was composed of 4 g tuna oil, 4 mL deionized water, and 2500 U *C. rugosa* lipase, and the reaction was carried out at 35°C with stirring (500 rpm) for 16 h. After the reaction, glycerides extracted with *n*-hexane were named as *Can.*-treated oil. The *Can.*-treated oil (12.6 g) was applied to a silica gel 60 column (30 × 260 mm; Merck, Darmstadt, Germany). Triglycerides were eluted with benzene, and then diglycerides were eluted with a mixture of benzene/ethyl acetate (9:1, vol/vol). The yields of di- and triglycerides were 7.8 and 3.8 g, respectively.

Preparation of DHA and EPA and their ethyl esters. The *Can.*-treated oil (50 g, DHA 49%, EPA 8.1%) was ethylated in ethanol using sodium ethylate as a catalyst. The ethyl esters of DHA and EPA were purified by the preparative high-performance liquid chromatography (HPLC) as described in the previous section, except for using methanol as a mobile phase. The yield of ethyl esters of DHA and EPA were 18.8 and 2.9 g, respectively, and their purities, as analyzed by gas chromatography, were 90.5 and 91.5%, respectively. DHA and EPA were prepared by extraction with *n*-hexane after saponification of the ethyl esters.

Synthesis of mono-, di-, and triglycerides of DHA. Monoglyceride of DHA (monoDHA) was synthesized by incubating the reaction mixture containing 6 g ethyl DHA, 18 g glycerol, and 50 mg NaOH in 50 mL dimethyl formamide at 55°C for 5 h. The extracts with *n*-hexane were applied to a silica gel 60 column, and mono-DHA was eluted with a mixture of benzene/ethyl acetate (3:2, vol/vol) after washing with the solvent mixture of 4:1 (vol/vol). The yield was 4.8 g.

Di- and triglycerides of DHA (di- and triDHA, respectively) were synthesized enzymatically. The reaction mixture containing 8 g DHA, 2.3 g glycerol, and 0.25 mL of 5000 U/mL *Ch. viscosum* lipase was incubated at 50°C for 70 h. Di- and triDHA were purified by silica gel 60 column chromatography as described previously. The yields of di- and triDHA were 0.9 and 4.1 g, respectively.

Hydrolyses of oils with lipase. Unless otherwise specified, a reaction mixture containing 0.5 g oil and 400 U lipase in 3.5 mL of 50 mM acetate buffer (pH 5.6) was incubated at 35°C for 16 h with stirring at 500 rpm. After the reaction, 30 mL ethanol was added, and the amount of liberated fatty acids were measured by titration with 0.1 or 0.4 N KOH. The hydrolysis extent was measured from the acid value of the reaction mixture and the saponification value of the oils.

Analytical procedures. Lipase activity was measured by titrating fatty acids liberated from olive oil with 0.05 N KOH, as described previously (14). The reaction was carried out at 30°C for 60 min with stirring at 500 rpm. One unit of lipase was defined as the amount of that liberated 1 μmol of fatty acid.

The fatty acids in glycerides were methylated by ester exchange with sodium methylate, and analyzed by gas chromatography, according to our previous paper (11). The contents of mono-, di-, and triglycerides were analyzed with a thin-layer chromatography/flame-ionization detector analyzer (Iatroscan TH-10; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol). ^{1}H Nuclear magnetic resonance (NMR) spectrum of triglyceride was measured in $CDCl_3$ using tetramethylsilane as the internal standard with a JNM-EX270 spectrometer (JEOL Ltd., Tokyo, Japan).

Chemicals. Ethyl esters of unsaturated fatty acid, except for DHA and EPA, were purchased from Funakoshi Co. (Tokyo, Japan). The other chemicals used were of the reagent grade.

RESULTS

Hydrolysis of tuna oil with G. candidum *lipase.* Tuna oil (DHA30) and olive oil were hydrolyzed with *G. candidum* lipase. The hydrolysis degree of olive oil was 89%, although that of tuna oil was 31% (Fig. 1). Even if the lipase (400 U) was added in the 16-h reaction mixture, the hydrolysis degree was not increased. Thus, the inactivation of lipase was not a cause of the cessation of hydrolysis.

Because the hydrolysis of oil with lipase is an equilibrium reaction, it may be inhibited by accumulation of FFA in the reaction mixture. Therefore, we attempted to investigate the inhibition of the reaction by fatty acids (Table 1). The fatty acids were prepared from the 16-h hydrolyzates of olive oil and tuna oil with *G. candidum* lipase. Fatty acids from olive oil inhibited the hydrolyses of olive oil and tuna oil, but fatty acids from tuna oil did not inhibit them as much. These results showed that the accumulation of FFA was not a cause of the cessation of the hydrolysis of tuna oil at the level of *ca.* 30%.

Action of ethyl esters of PUFA. Generally, the activities of

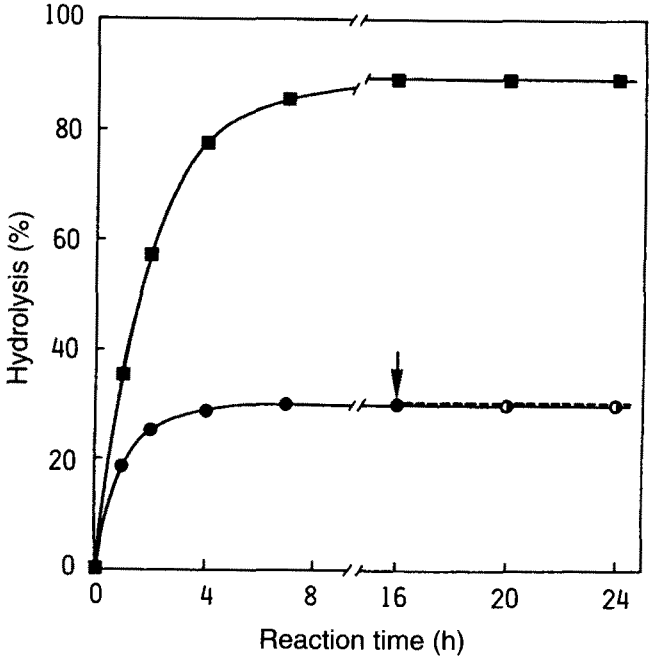

FIG. 1. Hydrolysis of olive oil and tuna oil with *Geotrichum candidum* lipase. Reaction conditions were described in the Materials and Methods section. Arrow indicates the addition of 400 units lipase in the reaction mixture. ■, Hydrolysis of olive oil; ●, hydrolysis of tuna oil; ○, hydrolysis of tuna oil after adding lipase.

lipase on PUFA esters are not so high (15). To clarify the activities of *G. candidum* lipase on PUFA esters, initial velocity and hydrolysis extent after 16-h reaction were investigated using ethyl esters of PUFA as substrates (Table 2). The extent of hydrolysis depended on the initial velocity. The lipase acted very well on oleic, linoleic, and α-linolenic acid esters, but not as well on γ-linolenic acid ester. The activities on arachidonic acid, EPA, and DHA esters were lower than that on γ-linolenic acid ester.

TABLE 1
Effects of Fatty Acids on Hydrolyses of Olive Oil and Tuna Oil with *Geotrichum candidum* Lipase[a]

Oil	Fatty acids from: Olive oil (mmol)	Fatty acids from: Tuna oil (mmol)	Hydrolysis (%)
Olive oil	0	0	92.2
	0.52	0	61.7
	1.56	0	46.6
	0	0.52[b]	87.5
	0	1.56[c]	82.0
Tuna oil	0	0	29.5
	0.49[b]	0	24.7
	1.48[c]	0	12.9
	0	0.49[b]	28.2
	0	1.48[c]	27.1

[a]Fatty acids were added in the basal reaction mixture containing 0.5 g oil, and the reaction was carried out as described in the Materials and Methods section.
[b]Corresponding to 30% hydrolysis of oils.
[c]Corresponding to 90% hydrolysis of oils.

TABLE 2
Activity of *Geotrichum candidum* Lipase on Various Unsaturated Fatty Acid Ethyl Esters

Substrate	Initial velocity[a] (%)	Hydrolysis[b] (%)
18:1n-9	100	77.2
18:2n-6	102	73.4
18:3n-3	135	75.7
18:3n-6	15.5	34.2
20:4n-6	8.8	14.5
20:5n-3	4.2	12.6
22:6n-3	1.0	4.5

[a]Reaction mixture containing 150 mg ethyl esters and 6–20 unit (U) lipase in 3.5 mL of 50 mM acetate buffer (pH 5.6) was incubated at 35°C for 1 h with stirring at 500 rpm. Initial velocity was expressed as the percentage of that observed on ethyl oleate.
[b]Ethyl ester was hydrolyzed under the same conditions as the above, except that 400 U lipase was used, and that reaction period was 16 h.

Tuna oil was fractionated to ten fractions by preparative HPLC, and the resulting triglycerides were specified by analytical HPLC (Fig. 2). The PUFA content decreased with increasing retention time. Using these triglycerides as substrates, the extent of their hydrolysis was investigated after 16-h incubation with *G. candidum* (Table 3). The extent of hydrolysis decreased with the increase in the PUFA content. From these results, it was suggested that the low hydrolysis extent of tuna oil with *G. candidum* lipase was caused by its low activity on PUFA esters, i.e., by its low hydrolysis rate.

Action on PUFA glycerides. Di- and triglycerides prepared from the *Can.*-treated oil were hydrolyzed with *G. candidum* lipase. The PUFA contents of di- and triglycerides were 74.5 and 63.0%, respectively. Interestingly, diglycerides were further hydrolyzed (27.0%) than triglycerides (19.1%), although their PUFA content was higher than that of triglycerides. The activities of *G. candidum* lipase on mono-, di-, and triDHA

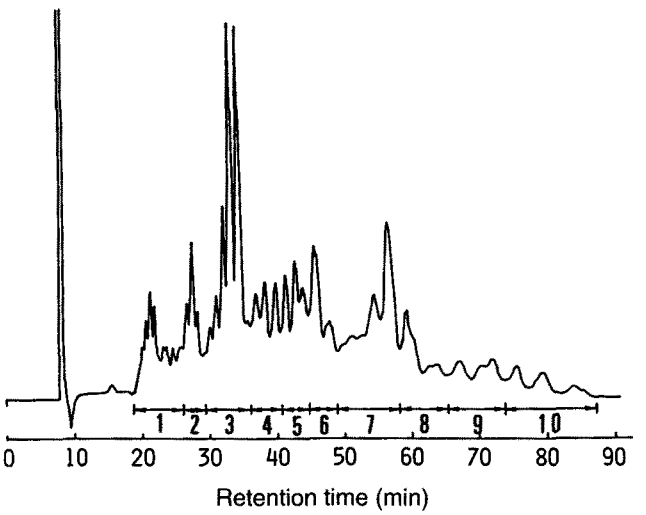

FIG. 2. Fractionation of tuna oil by high-performance liquid chromatography (HPLC). Tuna oil was fractionated into ten fractions, whose numbers are shown in the figure. The resulting triglycerides were specified by analytical HPLC, as in the Materials and Methods section.

TABLE 3
Hydrolysis of Tuna Oil Containing Different Amounts of Polyunsaturated Fatty Acid (PUFA) with *Geotrichum candidum* Lipase[a]

	Triglycerides[b]				
PUFA (%)	Fr. 1 (%)	Fr. 2 (%)	Fr. 5 (%)	Fr. 7 (%)	Fr. 10 (%)
EPA	19.4	14.7	8.1	5.7	2.3
DHA	53.2	47.8	33.8	26.8	14.0
Total[c]	80.6	67.1	49.9	36.7	20.8
Hydrolysis (%)	10.5	19.3	25.6	39.3	48.0

[a]The hydrolysis reaction was carried out as described in the Materials and Methods section; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
[b]The triglycerides indicated with fraction (Fr.) numbers are the same as those shown in Figure 2.
[c]Total PUFA include 20:4, 20:5, 22:3, 22:5, and 22:6.

TABLE 4
Activity of *Geotrichum candidum* Lipase on Mono-, Di-, and Triglycerides of DHA

		Glyceride composition[a]		
Substrate[b]	Hydrolysis (%)	TG (%)	DG (%)	MG (%)
MonoDHA	25.5	85.2	9.0	1.5
DiDHA	16.9	86.2	9.3	4.5
TriDHA	4.1	98.3	1.7	ND[c]

[a]In glyceride fraction after hydrolysis.
[b]MonoDHA, monoglyceride (MG) of DHA; diDHA, diglyceride (DG) of DHA; triDHA, triglyceride (TG) of DHA.
[c]ND, Not detected; see Table 3 for other abbreviations.

TABLE 5
Synthesis of Triglycerides from Mono- and Diglycerides with *Geotrichum candidum* Lipase

	Composition			
Reaction	TG (%)	DG (%)	MG (%)	FFA (%)
Glycerol + DHA (EPA)[a]				
Before reaction	ND[d]	ND	ND	100
After reaction	ND	ND	ND	100
MonoDHA + EPA[b]				
Before reaction	ND	ND	39.8	60.2
After reaction	28.6	6.4	5.3	59.7
DiDHA + EPA[c]				
Before reaction	ND	69.5	ND	30.5
After reaction	30.7	24.6	13.4	31.3

[a]Glycerol, 110 mg; DHA (or EPA), 390 mg; abbreviations as in Tables 3 and 4; FFA, free fatty acids.
[b]MonoDHA, 200 mg; EPA, 300 mg.
[c]DiDHA, 350 mg; EPA, 150 mg. The reaction mixture containing these substances and 400 units lipase in 3.5 mL of 50 mM acetate buffer (pH 5.6) was incubated at 35°C with stirring (500 rpm) for 16 h. Before and after the reaction, glycerides and fatty acids were extracted with ether, and their composition was analyzed by a thin-layer chromatography/flame-ionization detection analyzer.
[d]ND, not detected.

were investigated (Table 4). The enzyme acted on monoDHA better than on diDHA, and scarcely acted upon triDHA. In addition, triglycerides were the main component in glyceride fraction after the hydrolysis. The conversion of partial glycerides to triglyceride occurred even in the reaction mixture not containing acetate buffer, but did not occur when heat-denatured lipase was used. These facts indicated that the partial glycerides were converted to triglyceride by transacylation and/or condensation reaction(s) mediated by lipase. (We refer to the activity of lipase on mono-, di-, and triglycerides as glyceride specificity.)

Condensation of partial glycerides and PUFA. Any glyceride was not synthesized in the reaction mixture composed of glycerol, DHA (or EPA), and *G. candidum* lipase (Table 5). This result showed that the condensation reaction of glycerol and PUFA did not occur during the hydrolysis of tuna oil. MonoDHA and EPA or diDHA and EPA were reacted with the lipase for 16 h in the presence of 87.5% water. The molar ratios of monoDHA/EPA and diDHA/EPA were 1:2 and 1:1, respectively. The total amounts of FFA in both reactions were scarcely changed, and 72% of monoglyceride and 44% of diglyceride were converted to triglyceride. After the reaction, the fatty acid composition in glycerides was analyzed. The contents of EPA in the glycerides were 18.2 and 8.1% after the reactions of monoDHA and EPA, and diDHA and EPA, respectively. Thus, it was found that mono- or diDHA and EPA was esterified by condensation.

As acetic acid was present in that reaction mixture as a buffer component, it was necessary to confirm that acetoglyceride was not generated during the reaction. A proton signal of acetyl group ($\underline{CH_3}$–CO–) is generally observed as a single peak at 2.00–2.15 ppm of the ^1H NMR spectrum. This single peak was not observed in the spectrum of triglyceride synthesized in the reaction mixture containing mono- or diDHA and EPA (data not shown). This fact excluded the possibility that acetic acid used as buffer was incorporated into the triglyceride.

DISCUSSION

When tuna oil was hydrolyed with *G. candidum* lipase, PUFA (mainly DHA and EPA) were concentrated in glycerides, and triglycerides were the main components. We proved that fatty acid specificity and glyceride specificity of the lipase participated greatly in this hydrolysis reaction.

Condensation and transacylation during selective hydrolysis. TriDHA was generated by hydrolysis of mono- and diDHA (Table 4). This result shows that triDHA is formed by condensation between liberated DHA and partial glycerides and/or by transacylation between partial glycerides.

Table 5 shows that EPA was incorporated into the glyceride fraction by condensation between partial glycerides and EPA. If the formation of di- and triglycerides occurred only by condensation reaction with EPA, the contents of EPA in the glycerides from mono-DHA and EPA, and from diDHA and EPA are calculated to be 54.7 and 14.7%, respectively. However, the observed EPA contents were 18.2% (for the glycerides from monoDHA and EPA) and 8.1% (for the glycerides from diDHA and EPA), and they were lower than the calculated values.

These results suggested that the conversion of partial glycerides to triglycerides occurred not only by condensation but by transacylation, even in the reaction containing large amount of water.

Reaction mechanism of selective hydrolysis. When tuna oil was hydrolyzed with *G. candidum* lipase, the resulting glycerides contained high concentration of PUFA (mainly DHA and EPA), and triglycerides were the main component. This phenomenon can be explained by the following reaction mechanism; (i) The lipase has fatty acid specificity. Fatty acids, except PUFA (mainly palmitic and oleic acids), are released from tuna oil, and partial glycerides are generated. (ii) Parallel to the hydrolysis of the resulting partial glycerides, condensation reaction occurs between partial glycerides and FFA, and transacylation also occurs between partial glycerides. (iii) The ester hydrolysis rates of fatty acids except for PUFA are faster than those of PUFA. In addition, the activities on PUFA glycerides are mono- > di- > triglycerides, and PUFA triglycerides are barely hydrolyzed. These specificities of the lipase result in accumulation of PUFA-rich glycerides (mainly triglycerides) in the reaction mixture hydrolyzed tuna oil.

Tanaka *et al.* (16) synthesized the mixture of four kinds of triglycerides with a fatty acid composition of oleic acid and DHA (TG-DDD, TG-DDO, TG-DOO, and TG-OOO), and hydrolyzed the mixture with *C. rugosa* lipase. The result showed that the activity of the lipase was TG-OOO > TG-DOO > TG-DDO > TG-DDD. They defined this lipase property as triglyceride specificity. In our experiment, the extent of hydrolysis was decreased with the increase in the PUFA content (Table 3). This fact may suggest that *G. candidum* lipase also possesses triglyceride specificity, and that the specificity participates in the generation of PUFA partial glycerides from tuna oil.

Fatty acid specificity. The fatty acid specificity of *G. candidum* lipase was investigated (Table 2). The lipase did not act well on ethyl ester of γ-linolenic or arachidonic acid, or on ethyl esters of DHA nad EPA. Therefore, γ-linolenic and arachidonic acids also should be concentrated in glycerides by hydrolyzing oils containing them with this lipase.

Geotrichum candidum lipase showed low activities on fatty acids having an unsaturated bond between C_1 and C_7 from the carboxyl end. Each of these fatty acids has *cis* configuration, the main chain being bent (60°) at the unsaturated bond. A probable explanation for low enzyme activities, which we can give here in the light of the enzyme structure (17–19), is that the esters of these bent fatty acids cannot be accommodated in the active site of the enzyme. If any mutation expanding the active site cleft is introduced, the resulting enzyme may act on these substrates well.

REFERENCES

1. Bang, H.O., J. Dyerberg, and A.B. Nielsen, *Lancet 1*:1143 (1978).
2. Kromhout, D., E.B. Bosschietor, and C.D.L. Coulander, *New Engl. J. Med. 312*:1205 (1987).
3. Kanayasu, T., I. Morita, J. Nakao-Hayashi, N. Asuwa, C. Fujisawa, T. Ishii, H. Ito, and S. Murota, *Lipids 26*:271 (1991).
4. Philipson, B.E., D.W. Rothrock, W.E. Connor, W.S. Harris, and D.R. Illingworth, *New Engl. J. Med. 312*:1210 (1985).
5. Lee, T., R.L. Hoover, J.D. Williams, R.I. Sperling, J. Ravalese, B.W. Spur, D.R. Robinson, E.J. Corey, R.A. Lewis, and K.F. Austen, *Ibid. 312*:1217 (1985).
6. Bravo, M.G., R.J. Antueno, J. Toledo, M.E. Tomas, O.F. Mercuri, and C. Quintans, *Lipids 26*:866 (1991).
7. Stillwell, W., W. Ehringer, and L.J. Jenski, *Ibid. 28*:103 (1993).
8. Tsukuda, N., *Shokuhin Kogyo 9*:30 (in Japanese) (1985).
9. Hoshino T., T. Yamane, and S. Shimizu, *Agric. Biol. Chem. 54*:1459 (1990).
10. Tanaka, Y., J. Hirano, and T. Funada, *J. Am. Oil Chem. Soc. 69*:1210 (1992).
11. Shimada, Y., K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, *Ibid. 71*:951 (1994).
12. Sugihara, A., Y. Shimada, and Y. Tominaga, *Appl. Microbiol. Biotechnol. 35*:738 (1991).
13. Sugihara, A., Y. Shimada, M. Nakamura, T. Nagao, and Y. Tominaga, *Protein Engineering 7*:585 (1994).
14. Fukumoto, J., M. Iwai, and Y. Tsujisaka, *J. Appl. Microbiol. 10*:257 (1964).
15. Brockerfoff, H., *Biochim. Biophys. Acta 212*:92 (1970).
16. Tanaka Y., T. Funada, J. Hirano, and R. Hashizume, *J. Am. Oil Chem. Soc. 70*:1031 (1993).
17. Shimada, Y., A. Sugihara, Y. Tominaga, T. Iizumi, and S. Tsunasawa, *J. Biochim. 106*:383 (1989).
18. Shimada, Y., A. Sughihara, T. Iizumi, and Y. Tominaga, *J. Biochem. 107*:703 (1990).
19. Schrag, J.D., Y. Li, S. Wu, and M. Cygler, *Nature 351*:761 (1991).

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