Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase

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Three lipases, isolated previously in our laboratory, each with different fatty acid and positional specificities, and a known lipase from *Candida cylindracea* were screened for concentrating docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids in glycerides. *Geotrichum candidum* lipase was found to be suitable for their concentration in glycerides. Tuna oil was treated at 30°C with this lipase for 16 h, and 33.5% hydrolysis resulted in the production of glycerides containing 48.7% of DHA and EPA. The hydrolysis was not increased despite adding further lipase, so the glycerides were extracted, and the reaction was repeated. The second hydrolysis produced glycerides containing 57.5% of DHA and EPA in a 54.5% yield, with recovery of 81.5% of initial DHA and EPA. Of the total glycerides, 85.5% were triglycerides. These results showed that *G. candidum* lipase was effective in producing glycerides that contained a high concentration of polyunsaturated fatty acids in good yield.

KEY WORDS: DHA, enrichment, EPA, *Geotrichum candidum* lipase, hydrolysis, tuna oil.

Since epidemiologic studies were reported by Bang *et al.* (1), n-3 polyunsaturated fatty acids (PUFA) have become potential pharmaceutical substances. Especially, eicosapentaenoic (20:5, EPA) and docosahexaenoic (22:6, DHA) acids play a role in the prevention of a number of human diseases, including cardiovascular diseases (2–4), inflammation (5) and cancer (6,7). These physiological functions drew attention to the acids, and PUFA-rich oil was produced as a food material by the method of winterization (8). However, the yield was low, and the content of PUFA was less than 40%.

Lipases display two different specificities, namely fatty acid specificity and positional specificity. Lipases that act well on middle- and/or long-chain fatty acid esters are known to occur in nature (9). If there is a lipase that does not digest PUFA esters, PUFA will be concentrated in glycerides by hydrolyzing PUFA-containing oil with that lipase. Actually, *Candida cylindracea* lipase showed low reactivity on DHA esters, and glyceride oil containing 53% of DHA was produced by hydrolyzing tuna oil with this lipase (10). But EPA was not enriched in the oil with the lipase. Furthermore, because this enzyme also hydrolyzed DHA ester slightly, the recovery of DHA in glycerides was not good. If tuna oil is hydrolyzed with a lipase that shows lower reactivity on DHA and EPA esters than *C. cylindracea* lipase, PUFA-rich glycerides should be effectively produced. Therefore, the reactivities of three lipases, isolated in our laboratory (11–13), on PUFA esters were investigated and compared with that of *C. cylindracea* lipase.

In this paper, we describe how DHA and EPA can be effectively concentrated in glycerides with *Geotrichum candidum* lipase, whose reactivities on DHA and EPA esters are very low.

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MATERIALS AND METHODS

Lipases. The lipases from *G. candidum* (11), *Rhizopus delemar* (12) and *Fusarium heterosporum* (13) were prepared as reported before. Ammonium sulfate was added to the culture filtrate to give 80% saturation, and the resulting precipitates were dialyzed against water. *Candida cylindracea* lipase (Lipase-OF) was a gift from Meito Sangyo Co. (Nagoya, Japan).

Oil. Tuna oil, DHA30 (saponification value, 184; acid value, 0.05), refined by Maruha Co. (Tokyo, Japan), was used.

Hydrolysis of tuna oil with lipases. Unless otherwise specified, a reaction mixture containing 2 g tuna oil, 400 U lipase and 2 mL deionized water was incubated at 30°C for 16 h with stirring at 500 rpm. After the reaction, 20 mL ethanol was added, and the acid value was measured by titrating with 0.4 N KOH. The extent of hydrolysis was measured from the acid value of the reaction mixture and the saponification value of the original oil.

Fractionation of glycerides and free fatty acids (FFA) in reaction mixtures. Glycerides were extracted 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 with 100 mL *n*-hexane after adding 30 mL of 2 N HCl.

Analysis. Lipase activity was measured by titrating fatty acids liberated from olive oil (Wako Pure Chemical Ind., Osaka, Japan) 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 activity was defined as the amount that liberated 1 μmol of fatty acid per min.

Fatty acids in glycerides were methylated by ester exchange with sodium methylate, and free FFAs were esterified with gaseous HCl–methanol. These methyl esters were analyzed by gas chromatography. A Hewlett-Packard 5890 gas chromatograph (Avondale, PA) equipped with a flame-ionization detector and DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) was used. The column temperature was raised from 150 to 210°C at 2°C/min, and the temperatures of injector and detector were set at 250°C. The carrier gas was helium at a flow rate of 80 mL/min. Fatty acids were identified by comparison with standards.

The contents of mono-, di- and triglycerides (MG, DG and TG, respectively) in the glyceride fraction were analyzed with a TLC/FID analyzer (Iatroscan TH-10; Iatoron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7).

RESULTS AND DISCUSSION

Screening of a suitable enzyme for enrichment of PUFA. Tuna oil was hydrolyzed with several lipases according to the Materials and Methods section, and the fatty acid composition of FFA was then analyzed by gas chroma-

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tography. To compare the reactivities of lipases on fatty acid esters, the hydrolysis efficiency (H) of a lipase on each fatty acid ester was calculated according to the following formula:

$$H = F_f/F_g \quad [1]$$

where F_f and F_g are the contents (%) measured by gas chromatography, of a particular fatty acid in FFA after hydrolysis and in the original tuna oil before hydrolysis, respectively. The ester bond of a fatty acid with an H value less than 1 is less susceptible to hydrolysis by the lipase. Thus, by using the selective specificity of a lipase, certain fatty acids can be enriched in glycerides. Table 1 shows the hydrolysis efficiencies for the main fatty acids contained in tuna oil. *Candida cylindracea* lipase was found to be effective for the enrichment of DHA in glycerides but ineffective for EPA, whereas *R. delemar* lipase was not effective for the concentration of DHA, compared with *C. cylindracea* lipase, and cannot be used for the concentration of EPA. In contrast, *F. heterosporum* lipase was less effective on palmitic acid ester but more effective on oleic acid ester, compared with *C. cylindracea* lipase. It was also useful for the enrichment of EPA and DHA. Moreover, *G. candidum* lipase hydrolyzed palmitic and oleic acid esters more effectively than *F. heterosporum* and *C. cylindracea* lipases, and less effectively hydrolyzed EPA and DHA esters.

Table 2 shows the fatty acid composition of glycerides derived by treatment with *G. candidum* or *C. cylindracea* lipase. DHA was enriched by *C. cylindracea* lipase, but EPA was not. On the other hand, *G. candidum* lipase enriched both DHA and EPA.

Reaction conditions for concentrating PUFA. Figure 1 shows the relationships between the lipase amount and the extent of hydrolysis and the concentration ratios of DHA and EPA in the glycerides. The concentration ratios of DHA and EPA depended on the hydrolysis extent, and they did not increase above 80 U of lipase per gram of the reaction mixture. Therefore, the extent of tuna oil hydrolysis was used as an index for the enrichment of PUFA in the following experiments.

The effects of water content on the hydrolysis were investigated with 200 U of lipase per gram of oil or 100 U of lipase per gram of the reaction mixture (Table 3). When tuna oil was treated with 200 U of lipase per gram of oil, the highest hydrolysis level (35.4%) was attained at a water content of 50%. On the other hand, when the oil was treated with 100 U of lipase per gram of the reaction mixture, the highest hydrolysis levels (35.4–36.3%) were obtained at water contents of 50–70%. The effects of reaction temperature on the hydrolysis were also examined (data not shown), and the optimum temperature range was 30–35°C. In view of these results, the following experiments were carried out at 30°C with 200 U of lipase per gram of the reaction mixture containing 50% water.

Time course of tuna oil hydrolysis. Figure 2 shows the time course of hydrolysis and the accompanying concentration ratios of DHA and EPA in the glycerides. The hydrolysis of tuna oil and the concentration ratios of PUFA reached maxima after 7 h. The hydrolysis after 24 h was 33.8%, and the contents of DHA and EPA were 39.1 and 9.5%, respectively.

Enrichment of PUFA by repeated hydrolysis. Tuna oil (2 g) was hydrolyzed in a 4-g reaction mixture with 400 U of *G. candidum* lipase. The hydrolysis was 33.5% after 16 h of reaction. The same amount of lipase was again added to the reaction mixture, and the reaction was continued for another 60 h, but hydrolysis was not increased (34.3%).

To elevate the extent of hydrolysis, the glycerides were extracted from the lipase-treated oil, and the hydrolysis was repeated under the same conditions as described in the Materials and Methods section (Table 4). The repeated hydrolysis with *G. candidum* lipase elevated the hydrolysis level and produced glycerides containing 57.5% of DHA and EPA in 54.5% yield. In comparison with the single hydrolysis with *C. cylindracea* lipase, the yield of glycerides was improved by 6.6%, and the recovery of DHA and EPA was increased by 11.5%, while the total content of DHA and EPA was almost the same or only a little higher.

The repeated hydrolysis with *C. cylindracea* produced glycerides that contained 65.6% of DHA and EPA in 34.5% yield, and the recovery of DHA and EPA was 58.7%. The glycerides obtained by treatment with *C. cylindracea* lipase were hydrolyzed with *G. candidum* lipase. This treatment produced a mixture of glycerides containing almost the same content of DHA and EPA (64.2%) as that obtained by repeated hydrolysis with *C. cylindracea* lipase. The yield of glycerides by the treatment was better by 5.0%, and the recovery of DHA and EPA was

TABLE 1

Hydrolysis Efficiencies of Several Lipases on Ester Bonds of Main Fatty Acid Constituents of Tuna Oil

Fatty acid	Lipase			
	C. cylindracea[a]	*G. candidum*[b]	*R. delemar*[c]	*F. heterosporum*[d]
16:0	1.46	1.84	1.29	1.37
18:1	1.40	1.72	1.42	1.64
20:5	1.02	0.60	1.19	0.87
22:6	0.39	0.21	0.45	0.32

[a]*Candida cylindracea.*
[b]*Geotrichum candidum.*
[c]*Rhizopus delemar.*
[d]*Fusarium heterosporum.*

TABLE 2

Fatty Acid Compositions of Glycerides Derived by Hydrolysis of Tuna Oil with *Geotrichum candidum* or *Candida cylindracea* Lipase

Fatty acid	Original oil	Treated oil[a]	
		C. cylindracea[b]	*G. candidum*[c]
16:0	15.7	8.8	11.8
16:1	5.3	3.5	3.4
18:0	4.3	2.9	4.4
18:1	13.6	8.5	8.2
20:4	2.4	3.2	2.7
20:5	8.2	8.0	9.5
22:3	2.1	3.4	2.7
22:5	1.8	2.4	2.0
22:6	30.3	47.9	39.1
Hydrolysis	—	48.7%	33.5%

[a]Glycerides derived by hydrolysis.
[b]*Candida cylindracea.*
[c]*Geotrichum candidum.*

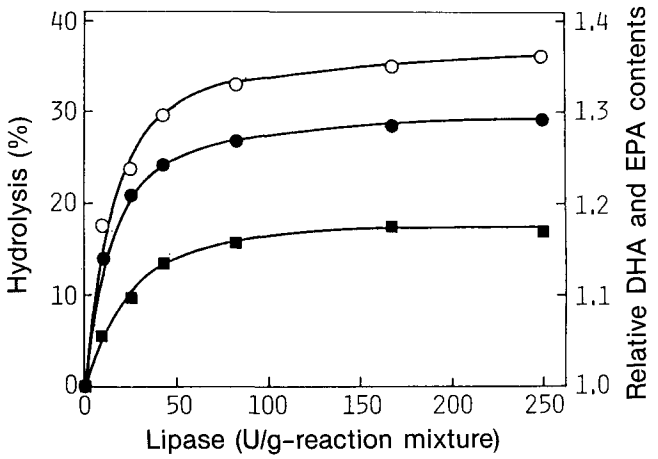

FIG. 1. Effect of the amount of *Geotrichum candidum* lipase on the hydrolysis of tuna oil. The docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contents in glycerides were expressed relative to those in the original oil, which were 30.3 and 8.2%, respectively; ○, hydrolysis of tuna oil; ●, enrichment of DHA in glycerides; ■, enrichment of EPA in glycerides.

TABLE 3

Effect of Water Content on Hydrolysis of Tuna Oil with *Geotrichum candidum* Lipase

Water content (%)	Composition of reaction mixture			Hydrolysis (%)
	Oil (g)	Water (mL)	Lipase (U)	
20	3.2	0.8	400[a]	28.5
			640[b]	30.2
30	2.8	1.2	400[a]	30.4
			560[b]	31.4
40	2.4	1.6	400[a]	31.3
			480[b]	33.5
50	2.0	2.0	400[a,b]	35.4
70	1.2	2.8	240[b]	24.2
			400[a]	36.3
90	0.4	3.6	80[b]	16.1
			400[a]	25.2

[a]Hydrolysis with 100 U of lipase per gram of the reaction mixture.
[b]Hydrolysis with 200 U of lipase per gram of oil.

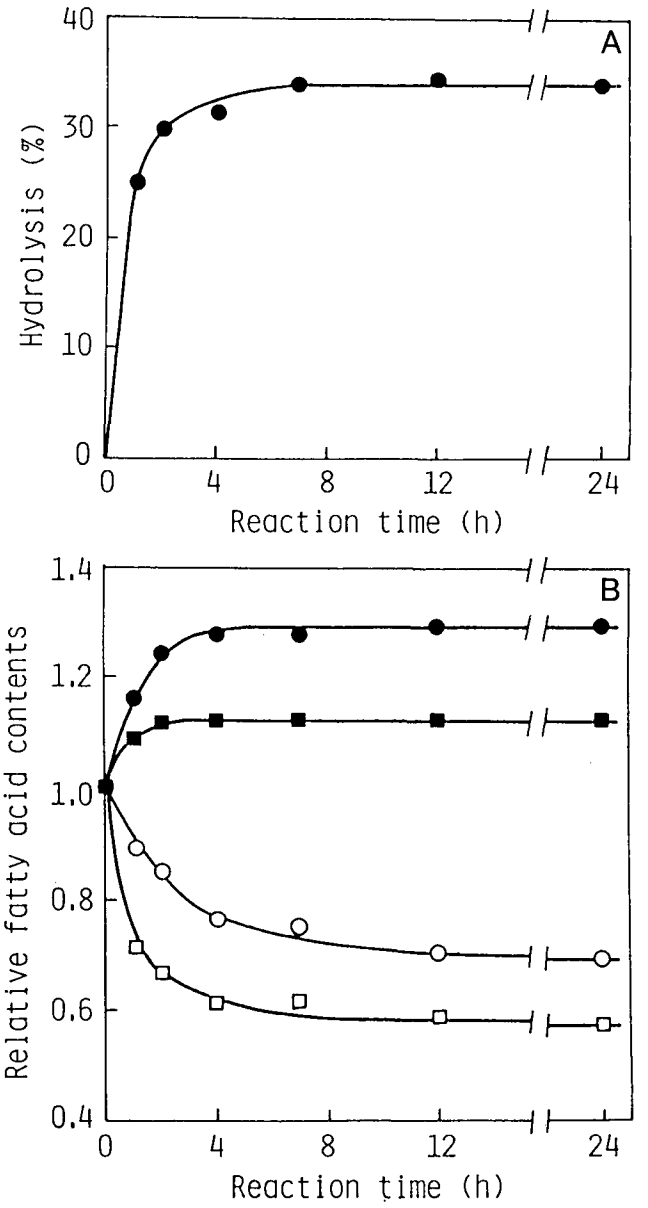

FIG. 2. Time course of the hydrolysis of tuna oil with *Geotrichum candidum* lipase (A) and the main fatty acid contents of glycerides after the hydrolysis (B). The fatty acid contents in glycerides were expressed relative to those in the original oil. Fatty acid contents in the original oil: palmitic acid (○), 15.7%; oleic acid (□), 13.6%; eicosapentaenoic acid (■), 8.2%; docosahexaenoic acid (●), 30.3%.

higher by 7.1%, compared with the repeated treatment with *C. cylindracea* lipase. These results showed that *G. candidum* lipase was more effective for the enrichment of PUFA in good yield than *C. cylindracea* lipase.

Table 5 shows the contents of TG, DG and MG in glycerides produced as described above. The results showed that TG content in glycerides produced with *G. candidum* lipase was higher, and DG and MG contents were lower, compared with those in glycerides produced with *C. cylindracea* lipase.

TABLE 4

Enrichment of DHA and EPA in Glycerides Derived by Hydrolyzing Tuna Oil with *Geotrichum candidum* or *Candida cylindracea* Lipase

Treatment	Yield (%)				Content[a] (%)		
	Gly[b]	DHA[c]	EPA[c]	DHA + EPA[c]	DHA	EPA	DHA + EPA
G. candidum	66.5	84.2	84.0	84.2	38.4	10.3	48.7
G. candidum + *G. candidum*	54.5	83.9	72.8	81.5	46.6	10.9	57.5
G. candidum + *C. cylindracea*	46.4	77.7	58.9	73.7	50.8	10.4	61.2
C. cylindracea	47.9	77.0	44.6	70.0	48.7	7.6	56.3
C. cylindracea + *G. candidum*	39.5	72.8	39.9	65.8	55.9	8.3	64.2
C. cylindracea + *C. cylindracea*	34.5	66.5	30.0	58.7	58.5	7.1	65.6

[a]Contents of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in glycerides after hydrolysis.
[b]Glycerides, calculated from the extent of hydrolysis.
[c]Values recovered in glycerides.

TABLE 5

Contents of Triglycerides (TG), Diglycerides (DG) and Monoglycerides (MG) in Glycerides Derived by Hydrolyzing Tuna Oil with *Geotrichum candidum* or *Candida cylindracea* Lipase

Treatment	Content (%)		
	TG	DG	MG
G. candidum	91.4	7.1	1.5
G. candidum + *G. candidum*	85.5	12.2	2.3
G. candidum + *C. cylindracea*	81.5	16.3	2.2
C. cylindracea	88.5	10.1	1.5
C. cylindracea + *G. candidum*	76.6	19.9	3.5
C. cylindracea + *C. cylindracea*	74.2	21.6	4.3

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. Iwai, M., and Y. Tsujisaka, in *Lipases*, edited by B. Borgstrom, and H.L. Brockman, Elsevier, Amsterdam, 1984, pp. 443–469.
10. Tanaka, Y., J. Hirano and T. Funada, *J. Am. Oil Chem. Soc. 69*:1210 (1992).
11. Tsujisaka, Y., M. Iwai and Y. Tominaga, *Agr. Biol. Chem. 37*:1457 (1973).
12. Iwai, M., and Y. Tsujisaka, *Ibid. 38*:1241 (1974).
13. Shimada, Y., C. Koga, A. Sugihara, T. Nagao, N. Takada, S. Tsunasawa and Y. Tominaga, *J. Ferment. Bioeng. 75*:349 (1993).
14. Fukumoto, J., M. Iwai and Y. Tsujisaka, *J. Appl. Microbiol. 10*:257 (1964).

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