Enrichment of Arachidonic Acid: Selective Hydrolysis of a Single-Cell Oil from *Mortierella* with *Candida cylindracea* Lipase

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ABSTRACT: Three lipases, isolated previously in our laboratory, and a known lipase from *Candida cylindracea* were screened for the enrichment of arachidonic acid (AA). The enzyme from *C. cylindracea* was the most effective for the production of oil with high concentration of AA. When a single-cell oil from *Mortierella alpina*, containing 25% AA, was hydrolyzed with this lipase for 16 h at 35°C, the resulting glycerides contained 50% AA at 52% hydrolysis. After this, no further hydrolysis occurred, even with additional lipase. However, when the glycerides were extracted from the hydrolyzate and were hydrolyzed again with new lipase, the resulting oil contained 60% AA, with a recovery of 75% of its initial AA content. Triglycerides were the main components of the resulting oil. The release of each fatty acid from the oil depended on the hydrolysis rate of its ester. The fatty acid, whose ester is the poorest substrate for the enzyme, is concentrated in the glycerides.
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KEY WORDS: Arachidonic acid, *Candida cylindracea* lipase, enrichment, fatty acid specificity, selective hydrolysis, single-cell oil from *Mortierella*.

Arachidonic acid (AA, 20:4n-6) is a rare fatty acid of potential pharmaceutical value and is a precursor of local hormones, prostaglandins, leukotrienes, and thromboxanes involved in the AA cascade (1,2). AA is also a component of human milk. Therefore, high concentration of AA is desired in powdered milk for infants. A method to concentrate polyunsaturated fatty acids (PUFA) in glycerides recently has been developed by the hydrolysis of natural oil with lipase. This method is referred to as selective hydrolysis (3). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and γ-linolenic acid (GLA) can be concentrated by this method (4–7), but the enrichment of AA has not yet been reported.

It is important to know the fatty acid specificity of lipase to enrich a specific fatty acid by selective hydrolysis (4,5,7). We recently proposed a new method to evaluate the fatty acid specificity of not only nonspecific, but also 1,3-positional specific, lipases by using an interesterified oil, in which the fatty acids were distributed randomly on the triglyceride molecules (7). The fatty acid specificities of four lipases were investigated by this method, and *Candida cylindracea* lipase was found to be the most effective to concentrate AA in glycerides.

In this paper, we describe a method to concentrate AA in glycerides by selective hydrolysis with *C. cylindracea* lipase, and point out that the release of fatty acids from the oil depends on the fatty acid specificity of the enzyme.

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MATERIALS AND METHODS

Lipases. Lipases from *Geotrichum candidum* (8), *Rhizopus delemar* (9), and *Fusarium heterosporum* (10) were prepared as reported previously. 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). The lipases from *R. delemar* and *F. heterosporum* are 1,3-positional specific, and those from *C. cylindracea* and *G. candidum* are nonspecific.

Oils. Sardine oil (EPA 18%) and tuna oil (DHA 30%) were obtained from Maruha Co. (Tokyo, Japan). Linseed oil was purchased from Yamakei Sangyo Co. (Osaka, Japan). A single-cell oil from *Mortierella alpina*, TGA-25 (AA 25%) (11), and SOS fat (1,3-distearoyl-2-oleoyl-*rac*-glycerol, purity 69%) were gifts from Suntory Co. (Osaka, Japan) and Fuji Oil Co. Ltd. (Osaka, Japan), respectively.

Random esterification. The arrangement of fatty acids in the triglyceride molecule was randomized by interesterification as described in our previous paper (7). The oil mixture (10 g sardine oil, 10 g tuna oil, 10 g linseed oil, and 20 g TGA-25) was interesterified by incubating at 65°C with stirring in the presence of 0.5% Na–methylate for 30 min. The resulting oil was extracted with 150 mL *n*-hexane after adding 50 mL water, and then the solvent was removed by evaporation. No partial glycerides were generated, and the fatty acid composition of the oil before and after the reaction was not changed. The recovery of interesterified oil was 96%.

Fractionation of glycerides and free fatty acids (FFA) in reaction mixture. Glycerides were extracted with 100 mL *n*-hexane under the alkaline condition after adding 0.5 N KOH (30% ethanol solution). FFA in the water phase were extracted with 100 mL *n*-hexane under acidic condition after adding 3 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 (12). The reaction was carried out at 35°C for 60 min with stirring at 500 rpm. One unit (U) of lipase activity was defined as the amount that liberated 1 μmol of fatty acid per minute.

Fatty acids in glycerides were methylated by ester exchange with Na–methylate, and FFA were esterified with gaseous HCl–methanol. These methyl esters 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, CA) as described previously (5).

The contents of mono-, di-, and triglycerides in the glyceride fraction 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).

Fatty acid specificity. Evaluation of fatty acid specificity of lipase with the interesterified oil was carried out as follows: A reaction mixture containing 1 g of interesterified oil (as described previously), 3 mL of 0.05 M acetate buffer (pH 5.6), and 100 U lipase was incubated at 35°C with stirring (500 rpm) for 30 min. After the hydrolysis, the FFA fraction was extracted with *n*-hexane, and its fatty acid composition was analyzed. The activity on a fatty acid ester was expressed according to the following formula:

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

where F_{ffa} and F_{ori} are the content (wt%), measured by gas chromatography, of a particular fatty acid in FFA after hydrolysis and in the oil before hydrolysis, respectively.

Hydrolysis of TGA-25 with lipase. Unless otherwise specified, a reaction mixture containing 2 g TGA-25, 2 mL deionized water, and 360 U lipase was incubated at 35°C for 16 h with stirring at 500 rpm. Ethanol (20 mL) was added after the reaction, and the acid value was measured by titrating with 0.5 N KOH. The extent of hydrolysis was calculated from the acid value of the reaction mixture and the saponification value of the original oil.

TABLE 1
Fatty Acid Specificities of Several Lipases Evaluated with Interesterified Oil[a]

Fatty acid	Lipase			
	Candida cylindracea	*Geotrichum candidum*	*Rhizopus delemar*	*Fusarium heterosporum*
16:0	75	75	96	102
16:1	214	217	105	110
18:0	48	26	91	92
18:1	100	100	100	100
18:2	130	151	96	94
18:3n-6	20	16	19	25
18:3n-3	198	139	100	96
20:4	17	11	34	38
20:5	22	13	35	38
22:6	13	7	10	16

[a]The activities on fatty acid esters were expressed as percentages of that on oleic acid ester. The fatty acid composition of the interesterified oil was as follows: 16:0 (11.8%), 16:1 (3.0%), 18:0 (4.5%), 18:1 (14.2%), 18:2 (13.0%), 18:3n-6 (2.6%), 18:3n-3 (13.4%), 20:4 (11.9%), 20:5 (5.5%), and 22:6 (9.3%).

RESULTS

Fatty acid specificities of several lipases. The fatty acid composition in FFA was analyzed after the interesterified oil was hydrolyzed at 35°C for 30 min with 100 U of lipase from *C. cylindracea, G. candidum, R. delemar,* or *F. heterosporum.* The extent of hydrolysis by the four lipases was almost the same (*ca.* 12%). The activities on a fatty acid ester were calculated according to Equation 1 and are shown in Table 1. These lipases acted well on the esters of palmitoleic, oleic (OA), linoleic (LA), and α-linolenic acids, but not on GLA, arachidonic acid (AA), EPA, and DHA. *Candida cylindracea* and *G. candidum* lipases did not act well on stearic acid (SA) ester.

Lipase suitable for enrichment of AA. A single-cell oil from *Mortierella* (TGA-25, 2 g) was hydrolyzed at 35°C for 16 h with 400 U of the four lipases. The contents of main fatty acids in the resulting glycerides are shown in Table 2. The extent of hydrolysis of TGA-25 by *C. cylindracea* and *G. candidum* lipases was 60 and 56%, respectively, and the content of AA in the resulting glycerides was increased from 25% to 51 and 47%, respectively. Furthermore, the recovery of AA in the

TABLE 2
Main Fatty Acid Contents in Glycerides Derived by Hydrolyses of Arachidonic Acid-Containing Oil (TGA-25) with Several Lipases

Lipase	Fatty acid content (%)						Hydrolysis (%)	Recovery (%)
	16:0	18:0	18:1	18:2	18:3[a]	20:4		
None	13.4	6.3	14.8	21.3	3.2	24.9	—	100
Candida cylindracea	4.6	3.2	6.3	9.2	4.5	51.0	59.8	82.3
Geotrichum candidum	5.9	5.8	5.5	8.3	4.3	47.3	55.7	84.1
Rhizopus delemar	12.3	5.3	14.2	21.0	3.9	28.0	36.4	71.5
Fusarium heterosporum	12.5	5.4	14.5	20.9	3.8	28.9	39.8	69.9

[a]γ-Linolenic acid.

glycerides was over 82%. On the other hand, when *R. delemar* and *F. heterosporum* lipases were used, the extent of hydrolysis was much lower and the content of AA was less than 30%. The recovery of AA was also lower, compared to that obtained with *C. cylindracea* and *G. candidum* enzymes. *Candida cylindracea* lipase was selected for further investigation.

Reaction conditions for concentrating AA. To decide on the amount of enzyme, TGA-25 (2 g) was hydrolyzed for 16 h with 10–270 U of lipase per gram of the reaction mixture. The extent of hydrolysis and the content of AA in glycerides increased with an increasing amount of enzyme (Fig. 1B). However, the recovery of AA in the glycerides was decreased by increasing the extent of hydrolysis (Fig. 1A).

The effect of water content on hydrolysis was investigated in the range of 20–90% at 90 U of lipase per gram of the reaction mixture. The optimum water content was 50–70%, and the extent of hydrolysis was 54%. The effect of temperature also was examined with the optimum temperature being 35°C.

In view of these results, the following experiments were carried out at 35°C with 90 U of lipase per gram of the reaction mixture of oil and water in the ratio of 1:1.

Time course of TGA-25 hydrolysis. Figure 2 shows the time course of TGA-25 hydrolysis and the accompanying relative concentration of fatty acids in glycerides. Hydrolysis of the oil reached 40% after 1 h and 55% after 20 h. Immediately after the hydrolysis, the contents of LA and OA in the glycerides decreased in that order, and that of AA increased. The content of palmitic acid (PA) decreased after a 30-min lag, and that of SA decreased gradually after an increase during the first 30 min. Furthermore, the content of GLA in the glycerides increased with hydrolysis and was slightly decreased after 20 h. The order of fatty acid release agreed with the fatty acid specificity of *C. cylindracea* lipase shown in Table 1.

Enrichment of AA by repeated hydrolysis. The extent of hydrolysis attained after 20 h was not increased, despite adding lipase and extending the reaction time, and the concentration ratio of AA did not increase either. In our previous report (5), we showed that repetition of hydrolysis was effective in increasing the extent of hydrolysis. Therefore, the glycerides extracted from the reaction mixture were repeatedly hydrolyzed under the conditions described in the Materials and Methods section.

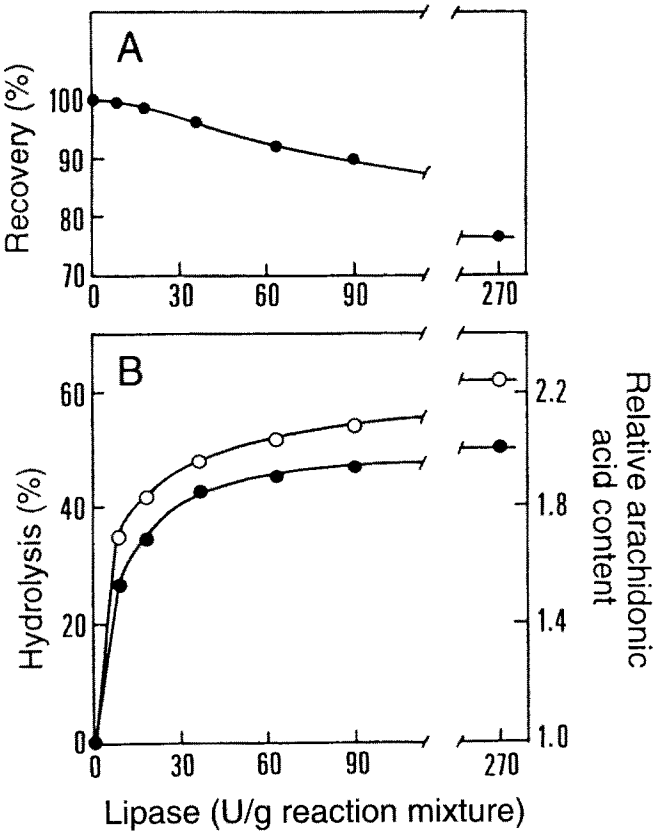

FIG. 1. Effect on the amount of *Candidum cylindracea* lipase on the hydrolysis of a single-cell oil [TGA-25 (Suntory Co., Osaka, Japan), a single-cell oil from *Mortierella alpina*]. A, Recovery of arachidonic acid (AA) in glycerides after hydrolysis. B, Extent of hydrolysis (○) and the concentration of AA in glycerides (●). The concentration of AA is expressed relative to the value in the original TGA-25 oil (24.9%).

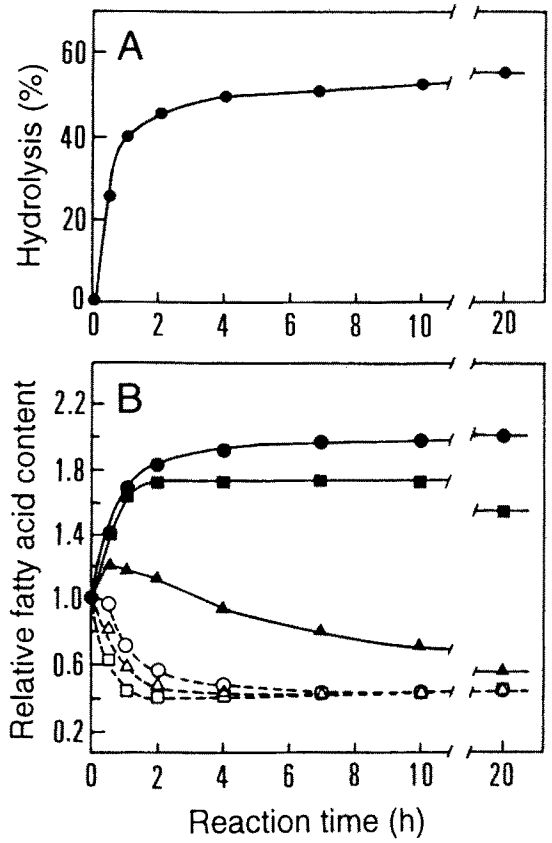

FIG. 2. Time course of hydrolysis of a single-cell oil, TGA-25, with *Candida cylindracea* lipase (A) and the main fatty acid content of glycerides after hydrolysis (B). The fatty acid contents in glycerides are expressed relative to those in the original oil. Fatty acid composition of the original oil is given in Table 2. ○, Palmitic acid; △, oleic acid; □, linoleic acid; ▲, stearic acid; ■, γ-linoleic acid; ●, AA. Other abbreviations as in Figure 1. See Figure 1 for company source.

TABLE 3
Main Fatty Acid Content in Glycerides Obtained by Repeated Hydrolysis

Treatment	Fatty acid content (%)						Hydrolysis (%)	Recovery (%)
	16:0	18:0	18:1	18:2	18:3[a]	20:4		
Untreated	13.4	6.3	14.8	21.3	3.2	24.9	—	100
First	5.7	3.6	6.7	9.6	4.7	49.5	52.4	94.6
Second	3.8	3.2	4.0	5.5	4.9	54.3	18.1	87.2
Third	2.4	2.3	2.5	3.3	5.2	59.7	17.2	75.0

[a]γ-Linolenic acid.

Table 3 shows the fatty acid compositions of glycerides obtained by repeat hydrolyses. The extent of the second and third hydrolyses was 18 and 17%, respectively. These values were lower than that of the first treatment (52%). The content of AA in glycerides was slightly increased by the repetition of hydrolysis, although recovery in the glycerides became lower.

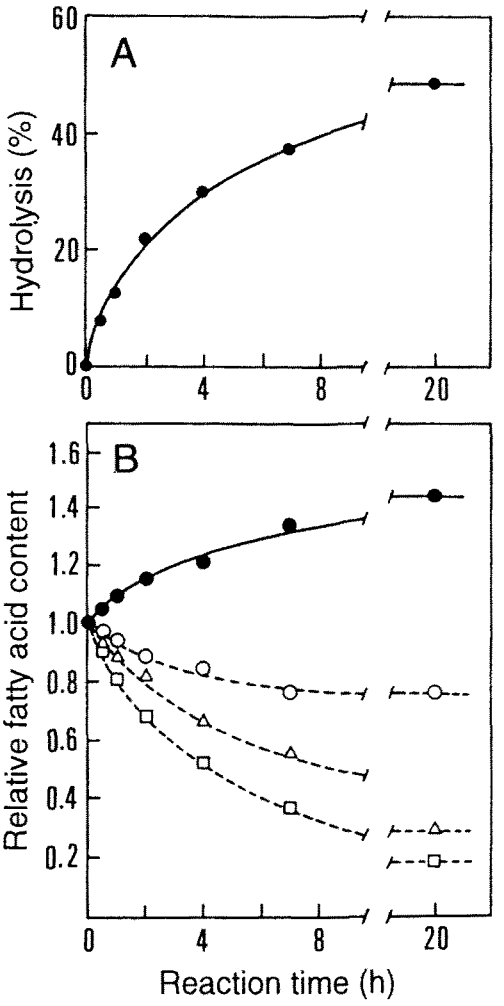

FIG. 3. Time course of hydrolysis of 1,3-distearoyl-2-oleoyl-*rac*-glycerol fat (Suntory Co., Osaka, Japan) with *Candida cylindracea* lipase (A) and the fatty acid content of the glycerides after hydrolysis (B). The fatty acid contents in glycerides are expressed relative to those in the original oil. Fatty acid composition of the original oil was stearic acid (59.4%, ●), oleic acid (32.5%, □), palmitic acid (3.4%, ○), and linoleic acid (3.2%, ▲).

The composition of the mono-, di, and triglycerides was analyzed after the repeat hydrolyses. Despite repeated hydrolysis, the content of triglyceride was scarcely changed; the first was 93%, the second, 91%, and the third, 93%.

Hydrolysis of SOS fat. To confirm that selective hydrolysis depends on fatty acid specificity, SOS fat was hydrolyzed at 37°C in a reaction mixture that contained 2 g fat, 2 mL water, and 70 U *C. cylindracea* lipase. The time course of the hydrolysis and the accompanying relative fatty acid concentration in the glycerides are shown in Figure 3. By increasing the extent of hydrolysis, the contents of LA, OA, and PA in the glycerides decreased in that order, and that of SA increased and attained 86.3% after 20 h. These results show that the order of fatty acid release agrees with the fatty acid specificity of the lipase, i.e., the hydrolysis rate of fatty acid ester (Table 1).

DISCUSSION

It recently has been reported that PUFA, such as DHA, EPA, and GLA, can be concentrated in glycerides by selective hydrolysis with lipase (4–7). In this paper, we describe a method to concentrate AA by hydrolyzing a single-cell oil from *Mortierella*. Selective hydrolysis is indeed useful for the industrial production of oil containing 45% DHA from tuna oil, and has been noted as a new method of oil processing.

To concentrate a specific fatty acid by selective hydrolysis, it is necessary to select lipases that act lightly on the ester of that fatty acid (4,5). Therefore, it is important to evaluate the fatty acid specificity of the lipase. The specificity has been investigated mainly by measuring the activity on simple triglycerides or methyl (ethyl) esters (13), or by analyzing the composition of fatty acids liberated from a natural oil used as a substrate (14). The evaluation by the former method is not precise because of the difference between the physical states of substrates—liquid and solid. By the latter method, the specificity of 1,3-specific lipase cannot be investigated because the fatty acids on triglyceride molecules in the natural oils are not randomly distributed. Therefore, we proposed a new method of investigating fatty acid specificity with an interesterified oil as the substrate (7), and we have adopted it in this study.

The recovery of AA upon hydrolyzing TGA-25 with *R. delemar* or *F. heterosporum* lipase was lower than that obtained with *C. cylindracea* or *G. candidum* enzyme (Table 2). This result is consistent with the fact that enzymes from *R. delemar* and *F. heterosporum* act well on AA ester as compared with those from *C. cylindracea* and *G. candidum* (Table 1). Furthermore, the order of fatty acid release depended on the fatty acid specificity of each specific lipase evaluated (Figs. 2 and 3).

When TGA-25 was hydrolyzed with *C. cylindracea* lipase, AA was concentrated in glycerides, but SA was not (Fig. 2). On the other hand, SA was effectively concentrated by hydrolyzing SOS fat with the same enzyme (Fig. 3). These facts show that the release of fatty acids from the oil depends on

the hydrolysis rate of the ester (Table 1). Therefore, the fatty acid, whose ester was the poorest substrate in the oil, was concentrated in the glycerides by the selective hydrolysis.

Triglycerides were the main components in the glycerides produced by selective hydrolysis of tuna oil with *G. candidum* lipase (5). We reported that the triglycerides were accumulated by the following mechanism: (i) Fatty acids, except PUFA, were preferentially released from the oil, and the PUFA–partial glycerides were generated. (ii) The PUFA–triglycerides were generated by condensation between the partial glycerides and PUFA and by transacylation between the partial glycerides. (iii) Because the PUFA–triglycerides formed were poor substrates for lipase, they were accumulated in the reaction mixture (15). As described in the text, the main components in the glycerides produced by hydrolyzing TGA-25 with *C. cylindracea* lipase were also triglycerides. Therefore, we presume that the selective hydrolysis of TGA-25 proceeded by the same mechanism as that of tuna oil.

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