Triglyceride Specificity of *Candida cylindracea* Lipase: Effect of Docosahexaenoic Acid on Resistance of Triglyceride to Lipase

Yukihisa Tanaka*, Tadashi Funada, Jiro Hirano and **Ron Hashizume**
Tsukuba Research Laboratory, NOF Corporation, 5-10 Tokodai, Tsukuba Ibaraki 300-26, Japan

Tuna oil was hydrolyzed with *Candida cylindracea* lipase. After 70% hydrolysis of the oil, the docosahexaenoic acid (DHA) content in the glyceride mixture [a mixture of TG (triglyceride), DG (diglyceride) and MG (monoglyceride)] was twice that of the original oil. DHA-rich TG and DG were observed, but DHA-rich MG was absent. *C. cylindracea* lipase seemed to have a "triglyceride specificity," and it favors TG without DHA over TG containing DHA. In accordance with this hypothesis, TG containing a mixture of oleic acid (OA) and DHA was synthesized and then hydrolyzed with *C. cylindracea* lipase. TGs in the hydrolysis product were fractionated and analyzed quantitatively by high-performance liquid chromatography. Four kinds of TGs were obtained. TG with three molecules of OA was hydrolyzed most easily. Increasing the DHA content of TG resulted in less hydrolysis of TG. The results suggested that *C. cylindracea* lipase had a TG specificity for the whole structure of TG in preference to the individual ester bonds; OA coexisting with DHA in TG was resistant to *C. cylindracea* lipase due to the TG structure.

KEY WORDS: *Candida cylindracea* lipase, DHA, HPLC, hydrolysis, triglyceride specificity.

Since the epidemiologic studies reported by Bang *et al.* (1), a great deal of research has focused on the roles of fatty acids. Docosahexaenoic acid (DHA) is the main material in the phospholipid of the retina (2) and the gray matter of the brain (3), and it is also thought to play an important role in the central nervous system.

As it is easier to purify DHA ethyl ester than DHA in triglyceride (TG) form, many researchers use the high-quality ethyl ester for animal and human studies. It has been reported, however, that DHA ethyl ester is not as easily incorporated into plasma TG as DHA into TG form (4). It is important to develop a method to economically produce DHA in the TG form.

The authors have tried to produce DHA-rich TG from fish oil and have observed that after hydrolysis of fish oil and DHA-rich tuna oil with *Candida cylindracea* lipase, the glyceride mixtures [mixtures of TG, diglyceride (DG) and monoglyceride (MG)] contained two or three times more DHA than the original oils (5).

In a trial of whale oil hydrolysis with 1,3-positional specific pancreatic lipase, DHA-rich 1,3-DG was obtained, as DHA is located in the 1,3-position of whale oil TG (6). According to Bottino *et al.* (6), after fish oil hydrolysis, DHA-rich 2-MG would be produced in the reaction mixture because nonpositional specific *C. cylindracea* lipase has fatty acid specificity, and DHA is hardly hydrolyzed (7). In most fish oil hydrolysis products, however, DHA-rich TG and DG were observed, whereas 2-MG was not (5). If lipase recognizes the individual ester bonds of DHA, saturated and monenoic acids in TG are hydrolyzed preferentially, regardless of the presence of DHA in TG. DHA-rich MG and DG are then obtained in the hydrolysis products. As DHA-rich TG was obtained and MG was absent, the results indicated that the lipase recognizes the whole TG structure. The results are difficult to explain in terms of fatty acid specificity, and another type of specificity is necessary. The authors defined this specificity as "triglyceride specificity."

In this report, that hypothesis is substantiated. TG with a fatty acid composition composed of a mixture of oleic acid (OA) and DHA was synthesized, hydrolyzed with *C. cylindracea* lipase and analyzed quantitatively by high-performance liquid chromatography (HPLC). The analysis by HPLC indicated that *C. cylindracea* lipase had TG specificity. TG-containing DHA was hydrolyzed with *C. cylindracea* lipase more slowly than TG not containing DHA.

As a result of this experiment, the authors were able to explain why even at high-percentage hydrolysis, DHA-rich TG was resistant to *C. cylindracea* lipase, and the largest component of the glyceride mixture was DHA-rich TG.

MATERIALS AND METHODS

Lipase. Candida cylindracea lipase was purchased from Meito Sangyo Co., Ltd. (Nagoya, Japan).

Synthesis of TG. TG with a fatty acid composition composed of OA and DHA was named the pseudo-fish oil (PFO). OA (99.9%) and glycerol were from NOF Corporation (Tokyo, Japan). DHA (92.3%) was purchased from Nippon Chemical Feed Co., Ltd. (Hakodate, Japan). PFO was synthesized chemically with glycerol and a free fatty acid (FFA) mixture of DHA and OA.

Measurement of lipase. The lipase activity was measured according to the Japanese Industrial Standard (JIS) method (8).

Hydrolysis reaction. Five grams of PFO and 5 g distilled water containing 500 unit/oil of lipase were mixed at 37°C, stirred at 500 rpm and bubbled with nitrogen gas to prevent oxidation. Methanol was added to stop the reaction, and the hydrolysis (%) [acid value (AV)/saponification value (SV)] was measured by titration with 0.1N ethanolic KOH solution.

Quantitative analysis of hydrolysis products. For quantitative analysis of the extent of hydrolysis (the contents of TG, DG, MG and FFA), the products were separated by thin-layer chromatograph (TLC), silica gel plate 5721 (Merck, Darmstadt, Germany) with $CHCl_3$/acetone (96:4, vol/vol), and the spots were measured with a dual-wavelength chromato scanner CS-930 (Shimadzu, Kyoto, Japan) at 465 nm.

Synthesis and fractionation of didocosahexaenoyl glycerol (DHA-DG) and monodocosahexaenoyl glycerol (DHA-MG). DHA-DG and DHA-MG were synthesized with DHA and glycerol. The products, with contained DHA-TG, DHA-DG, DHA-MG and DHA-FFA, were fractionated by centrifugal partition chromatography (CPC-MNF) (Sanki Engineering Ltd., Kyoto, Japan). The eluting solvent was *n*-hexane/85% ethanol. DHA-DG was fractionated in the normal ascending and the reversed descending fractions (9,10).

Analysis of fatty acid compositions. The hydrolysis products were developed on TLC. TG, DG, MG and FFA

*To whom correspondence should be addressed.

were extracted with diethyl ether, then methylated with 14% borontrifluoride in methanol, and analyzed by gas chromatography. A Hewlett Packard (Palo Alto, CA) 5890 gas chromatograph equipped with a flame-ionization detector (FID) and the capillary column DB-WAX (30 m × 0.25 mm i.d.) (J&W Scientific, Folsom, CA) was used. The column temperature was raised from 150 to 210°C at 5°C/min. Injection and detector temperatures were 250°C. Fatty acids were identified by comparison of retention times with authentic standards.

Quantitative analysis of TG molecular species by HPLC. Four kinds of TGs were found in PFO. TGs that contained three molecules of DHA, two molecules of DHA, one molecule of DHA and no molecules of DHA were named TG-DDD, TG-DDO, TG-DOO and TG-OOO, respectively. The HPLC used for their analysis consisted of an L-6000 pump (Hitachi, Tokyo, Japan), an L-6200 intelligent pump (Hitachi) and an L-3300 RI monitor (Hitachi). The separations were performed on a packed column Wakosil-II 5C18 HG pre-column (4.6 mm × 10 mm) and main column (4.6 mm × 250 mm) (Wako Pure Chemical Industries Ltd., Osaka, Japan). The conditions were as follows: the eluting solvent was acetonitrile/ethanol/2-propanol (4:1:0.56, vol/vol/vol), the column oven temperature was 65°C and the flow rate was 1.1 mL/min. Four TGs were separated and identified by gas chromatography/mass spectrometry (GC/MS) (JMS DX 303, JEOL, Tokyo, Japan). TG on reversed-phase column with nonaqueous mobile phases elutes according to the equivalent carbon number (ECN) [ECN is defined as: ECN = CN − (2 × NDB), where CN is the carbon number and NDB is the number of double bonds]. The lowest ECN elutes first (11). The eluting order of TGs was, according to ECN: TG-DDD (ECN = 30), TG-DDO (ECN = 36), TG-DOO (ECN = 42) and TG-OOO (ECN = 48), and retention times were 6.9, 9.9, 15.0 and 22.6 min, respectively. Under these conditions, it was difficult to effectively separate isomers containing acyl groups at different positions.

Quantitative calibration curves for all four TGs were prepared with mixtures of TG-DDD and each of the other TGs (TG-DDO, TG-DOO and TG-OOO). The injected amount was compared with the monitored area ratio. Calibration curves between the actual amount ratio and the peak area ratio (RI monitor response) were obtained: $(A_2/A_1) = 0.8811(M_2/M_1) - 0.1007$ ($r = 0.9993$); $(A_3/A_1) = 0.8658(M_3/M_1) - 0.1991$ ($r = 0.9985$); and $(A_4/A_1) = 0.7599(M_4/M_1) + 0.05728$ ($r = 0.9996$); $A_1 \approx A_4$ were the actual amounts (mg), and $M_1 \approx M_4$ were the peak areas (%). M_1 and A_1 represent TG-DDD, M_2 and A_2 for TG-DDO, M_3 and A_3 for TG-DOO, and M_4 and A_4 for TG-OOO.

RESULTS AND DISCUSSION

Tuna oil (DHA, 25.1%) was hydrolyzed with *C. cylindracea* lipase. FFA in 70% hydrolysis product was removed by titration, and the DHA content of the glyceride mixture (containing TG, DG and MG) was determined to be 53.1%. The glyceride mixture contained mostly DHA-rich TG and DG, but not MG (5). The reason for this result was presumed to be that the reactivity of *C. cylindracea* lipase for TG containing DHA was less than that for TG that did not contain DHA. *C. cylindracea* lipase recognizes the whole TG structure in preference to the individual ester bonds of TG. Thus, the glyceride mixture derived from 70% tuna oil hydrolysis product contained DHA-rich TG (DHA content, 52.4%) and DG (55.3%). As tuna oil is composed of many kinds of fatty acids, there are numerous TG species in tuna oil. Although Wojtusik *et al.* (12) reported on the analysis of TG species in fish oil by HPLC, it is too complicated and difficult to apply their work to analysis of TG specificity of lipase. Therefore, we have synthesized TG containing a mixture of DHA and OA (named PFO) to simplify the structure of TG and its hydrolysis with *C. cylindracea* lipase.

Hydrolysis of PFO. PFO was hydrolyzed with *C. cylindracea* lipase (Fig. 1). The hydrolysis products were titrated at suitable times to calculate the degree of hydrolysis (%) and analyze the contents of TG, DG, MG and FFA (Fig. 2). Free glycerol was removed with water from the reaction mixture. TG was always higher than DG in the hydrolysis products, with only a minimum presence of MG.

The content of TG species in the original PFO was analyzed by HPLC. The ratio of TG-DDD/TG-DDO/TG-DOO/TG-OOO was found to be 20.3:25.4:20.7:33.7 (wt%). This ratio was obtained from the calibration curves (described in the Materials and Methods section), and the peak area (%) monitored by HPLC.

Quantitative changes of the four TG species were calculated according to the formula:

$$Wn = (A \times Bn)/Cn \quad [1]$$

where Wn equals the residual ratio of each TG molecular species in the TG fraction for the original PFO (%) (resistance of TG species to *C. cylindracea* lipase); A, the weight percent of the TG fraction in the PFO hydrolysis product at each hydrolysis rate (analysis was by dual-wavelength chromato scanner); Bn, the weight percent of

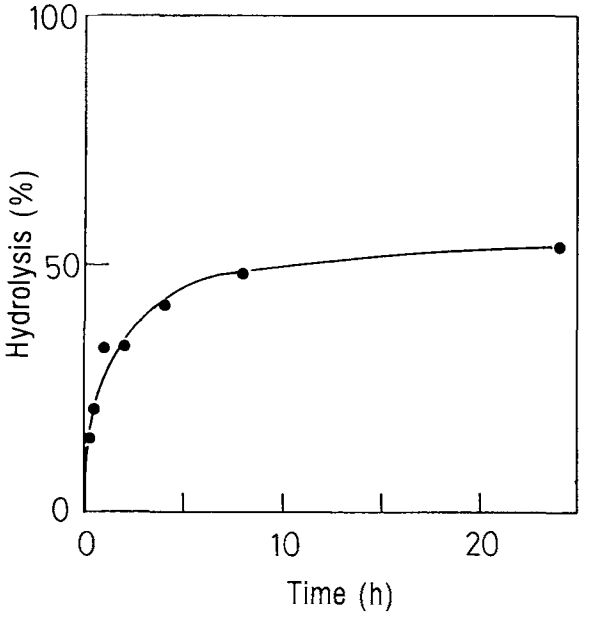

FIG. 1. Pseudo-fish oil was hydrolyzed with *Candida cylindracea* lipase at 37°C., stirred at 500 rpm. The ratio of oil and water was 1:1 (w/w).

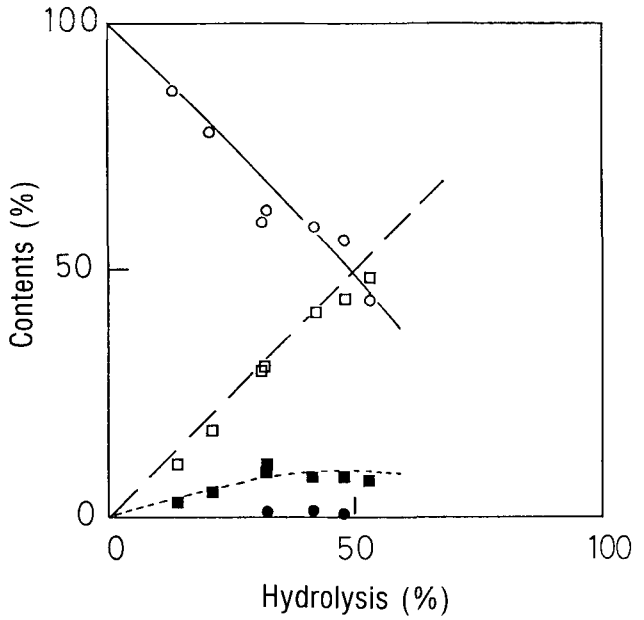

FIG. 2. Hydrolysis of pseudo-fish oil with *Candida cylindracea* lipase. ○, ■, ● and □ represent triglycerides, diglycerides, monoglycerides and free fatty acids, respectively, in the hydrolysis products.

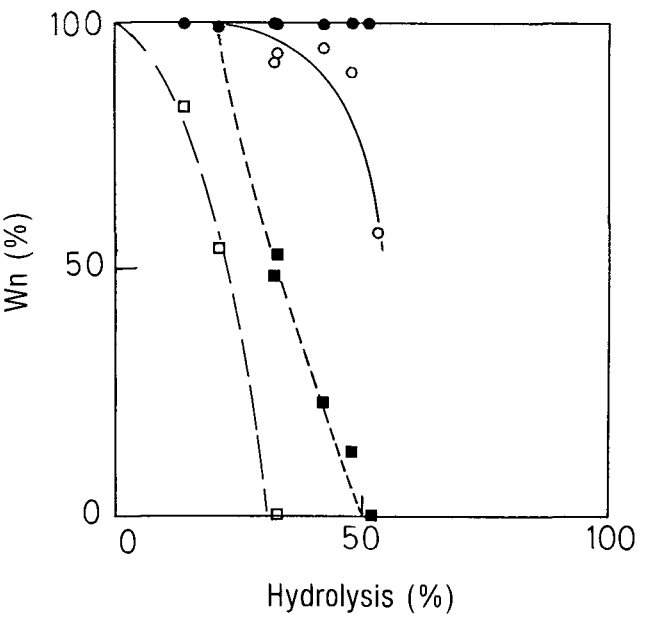

FIG. 3. The resistance of triglyceride (TG) molecular species to *Candida cylindracea* lipase. Wn is the ratio of each TG species in the hydrolysis products and TG in the original pseudo-fish oil. For n, see text. W_1●; W_2○; W_3■ and W_4□.

each TG species (TG-DDD, TG-DDO, TG-DOO and TG-OOO) in the TG fraction derived from the PFO hydrolysis product at the same hydrolysis rate selected at A; the weight percents of TG species were converted by using the calibration curves from the peak area percents monitored by HPLC; Cn, the weight percent of each TG species in the original PFO: C_1 was 20.3%, C_2 was 25.4%, C_3 was 20.7%, and C_4 was 33.7%; and n was $1 \approx 4$; W_1, B_1 and C_1 were for TG-DDD; W_2, B_2 and C_2 were for TG-DDO; W_3, B_3 and C_3 were for TG-DOO; and W_4, B_4 and C_4 were for TG-OOO.

TG-OOO was more easily hydrolyzed than any other TG. TG-DDD was the least easily hydrolyzed (Fig. 3). The resistance of the TGs to *C. cylindracea* lipase was TG-DDD > TG-DDO > TG-DOO > TG-OOO, with lipase activities for TG-OOO and TG-DDD being 84 u/mg and 1 u/mg, respectively. These results support the results of the HPLC analysis. Because TG-OOO and TG-DOO were hydrolyzed more easily than TG-DDO and TG-DDD, the DHA content in TG increased with advancing hydrolysis (Fig. 4). Because the nonpositional specific *C. cylindracea* lipase has fatty acid specificity, it does not hydrolyze DHA as well as OA in the TG (7). If OA in TG-DDO, TG-DOO and TG-OOO was hydrolyzed equally well, these TGs would disappear from the TG fraction of hydrolyzed PFO in a similar fashion. The result was different than the presumption. The hydrolysis rate depended mainly on the presence of the DHA molecule in TG.

Bottino *et al.* (6) reported that DHA has a terminal methyl group close to its carboxyl group, which protects against hydrolysis. The fatty acid composition probably makes a difference in the shape of a TG molecule. *Candida cylindracea* lipase may not be able to recognize OA due to the steric hindrance of DHA, and OA coexisting with DHA in TG was more resistant to *C. cylindracea* lipase than OA without DHA in TG. We defined this difference of activity to TG species as "triglyceride specificity."

The result was applied to the hydrolysis of tuna oil. After 70% hydrolysis, the DHA content in the glyceride mixture was 53.1% (5). TG that did not contain DHA was

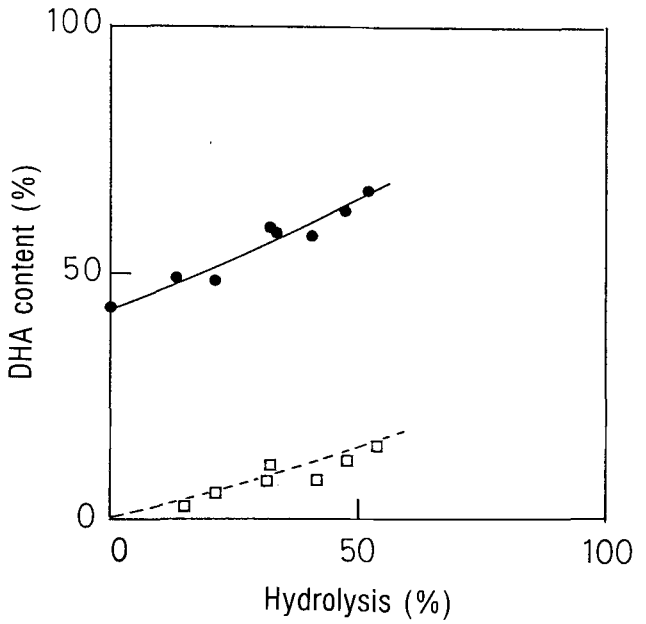

FIG. 4. Docosahexaenoic acid (DHA) content of triglyceride (●) and free fatty acid (□) in hydrolysis products, when pseudo-fish oil was hydrolyzed with *Candida cylindracea* lipase.

hydrolyzed in preference to TG with DHA. TG that contained one or two DHA molecules were subsequently hydrolyzed.

As a result, the DHA content in TG and DG of the glyceride mixture was about 50% (5). Because tuna oil contains little TG with three molecules of DHA, DG was mostly derived from TG that contained one molecule of DHA. As the lipase activities for DHA-DG and DHA-MG were 1.5 u/mg and 13 u/mg, respectively, DG derived from TG that contained two molecules of DHA was not hydrolyzed to any great extent. On the other hand, because MG is easily hydrolyzed, MG was not observed in the hydrolysis products after hydrolysis.

The DHA content of fish oil can be enriched by crystallization in acetone at −60°C. After this treatment, TG in tuna oil that does not contain DHA is removed while TG that contains DHA is enriched. Such tuna oil (*e.g.*, DHA 38.4%, eicosapentaenoic acid 10.5%) is therefore not suitable for further enrichment of DHA by hydrolysis with *C. cylindracea* lipase.

ACKNOWLEDGEMENT

The authors thank Liew Cheechin (Tohoku University) for his suggestions on the HPLC analysis.

REFERENCES

1. Bang, H.O., J. Dyerberg and A.B. Nielsen, *Lancet 1*:1143 (1971).
2. Anderson, R.E., *Exp. Eye Res. 10*:339 (1970).
3. Svennerholm, L., *J. Lipid Res. 9*:570 (1968).
4. Lawson, L.D., and B.G. Hughes, *Biochem. Biophys. Res. Commun. 152*:328 (1988).
5. Tanaka, Y., J. Hirano and T. Funada, *J. Am. Oil Chem. Soc. 69*:1210 (1992).
6. Bottino, N.R., G.A. Vandenburg and R. Reiser, *Lipids 2*:489 (1967).
7. Φyvind, L., and G. Lambersten, *Fette Seifen 88*:365 (1986).
8. Japanese Industrial Standards Committee, *Japanese Industrial Standard*, JIS K601 (1988).
9. Murayama, W., Y. Kosuge, N. Nakaya, Y. Nunoguchi, K. Nunoguchi, J. Cazes and H. Nunogichi, *J. Liq. Chromatogr. 11*:283 (1988).
10. Tanaka, Y., T. Funada, W. Murayama and Y. Kosuge, *J. Jpn. Oil Chem. Soc. (YUKAGAKU) 41*:23 (1992).
11. El-Hamdy, A.H., and E.G. Perkins, *J. Am. Oil Chem. Soc. 58*:867 (1981).
12. Wojtusik, M.J., P.R. Brown and J.G. Turcotte, *BioChromatography 3*:76 (1988).

[Received February 1, 1993; accepted July 2, 1993]