Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with *Candida cylindracea* Lipase

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

In an attempt to concentrate the content of DHA (docosahexaenoic acid) in a glyceride mixture containing triglyceride, diglyceride and monoglyceride, fish oil was hydrolyzed with six kinds of microbial lipase. After the hydrolysis, free fatty acid was removed and fatty acid components of the glyceride mixtures were analyzed. When the hydrolysis with *Candida cylindracea* lipase was 70% complete, the DHA content in the glyceride mixture was three times more than that in the original fish oil. The EPA (eicosapentaenoic acid) content became almost 70% of the original fish oil. Hydrolysis with other lipases did not result in an increase in the DHA content in the glyceride mixtures. Hydrolysis of DHA-rich tuna oil (DHA content is about 25%) with *Candida cylindracea* lipase resulted in 53% DHA in the glyceride mixture. The EPA content, however, remained close to that of the original tuna oil. In this report, the acyl chain specificity of lipases is evaluated in terms of hydrolysis resistant value (HRV). HRV is the ratio between the DHA contents in the glyceride mixture of hydrolyzed oil and original oil. HRV clearly indicates differences in hydrolysis between DHA and other fatty acids (*e.g.*, saturated and monoenoic acids).

KEY WORDS: *Candida cylindracea* lipase, concentration, DHA, EPA, fish oil, hydrolysis, tuna oil.

The n-3 family of polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been the focus of attention of late because of their clinical effects. Physiological studies have suggested that n-3 PUFA and n-6 PUFA (*e.g.*, γ-linoleic acid and archidonic acid) show competitive action against homeostasis. Cardiovascular disorders and related diseases may be due to an intake imbalance between n-3 and n-6 PUFAs.

As DHA is the main material in the phospholipids of the retina (1) and the brain (2), it is thought to play an important role in the central nervous system. As humans cannot synthesize DHA *in vivo*, the main source of DHA is in seafood. Of all the concentration methods tried, such as urea complexation and high-performance liquid chromatography (HPLC), only PUFA esters show promise. It is difficult to purify PUFA in the glyceride form efficiently and economically.

It has been reported that EPA and DHA ethyl esters are not as easily incorporated into plasma triglyceride (TG) as EPA and DHA in the TG form (3). Therefore, it is important to develop purification and concentration methods for PUFA in the glyceride form.

Lipases have both positional specificity (4) and acyl chain specificity (5). In a trial to hydrolyze whale oil with 1,3-specific pancreatic lipase (6) in an attempt to concentrate n-3 PUFAs in the 2-position of TG, the authors expected 1,3-specific lipase to hydrolyze the saturated and monoenoic acids that were placed in the 1,3-position, and thus PUFA would be concentrated as the 2-monoglyceride.

After 70% hydrolysis of fish oil and DHA-rich tuna oil with nonspecific *Candida cylindracea* lipase and subsequent FFA (free fatty acids) removal, the DHA contents in the glyceride mixture (including TG, diglyceride [DG] and monoglyceride [MG]) were 30% (fish oil) and 50% (tuna oil). As EPA was hydrolyzed more easily than DHA, the final EPA content was less than in the original oils. These results mean that *Candida cylindracea* lipase has acyl chain specificity and shows resistance to DHA in TG. This article reports on the use of lipases for DHA concentration.

MATERIALS AND METHODS

Lipases. Details on the lipases (and their characteristics) used in this article are presented in Table 1. These lipases were gifts from the manufacturer.

Fish oils. Fish oil and tuna oil refined by the NOF Corporation (Tokyo, Japan) were used. Fish oil contained 13.3% EPA, 8.9% DHA and 15.6% OA (oleic acid). Tuna oil contained 5.6% EPA, 25.1% DHA and 14.1% OA.

TABLE 1

Microbial Lipases and Their Characteristics

Organism	Optimal temperature (°C)	Positional specificity	Activity[a]	Manufacturer[b]
Candida cylindracea	30-40	None	70.0	Meito Sangyo
Aspergillus niger	30-40	1,3-	6.3	Amano
Pseudomonas sp.	40-65	None	5.5	Amano
Rhizopus delemar	30-45	1,3-	2.0	Tanabe
Rhizopus javanicus	30-45	1,3-	8.1	Amano
Chromobacterium viscosum	60-70	None	8.5	Asahi Chemical

[a]Activity (u/mg) was measured according to the Japanese Industrial Standards method. Substrate was fish oil.

[b]Amano Pharmaceutical Co., Ltd., Nagoya, Japan; Asahi Chemical, Tokyo, Japan; Meito Sangyo, Ltd., Nagoya, Japan; and Tanabe, Tokyo, Japan.

*To whom correspondence should be addressed.

Measurement of lipase activity. The lipase activity was measured according to the Japanese Industrial Standard (JIS) method (7), substituting fish oil for olive oil as substrate.

Hydrolysis reaction. Fish oil (tuna oil; 50 g) and 50 g of distilled water containing 200 units of lipase per 1 g of oil were mixed at 37°C, stirred at 500 rpm and bubbled with nitrogen gas to prevent oxidation. Methanol was added to the reaction mixtures and the hydrolysis (%) (ratio of acid value/saponification value) was measured by titration with 0.1 ethanolic KOH solution.

Analysis. After addition of 10 mL acetone and 10 mL hexane to 2 mL of the reacted mixture, the solution was titrated with 1/2 N methanolic KOH solution to remove FFA. After shaking and standing, the lower layer was removed and the upper layer was washed ten times with 20 mL distilled water. After evaporation of solvent and drying, the residue was esterified with borontrifluoride methanol agent and analyzed by gas chromatography. A Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and Carbowax 20M capillary column (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. Both injector and detector temperatures were 250°C. The carrier gas was helium at a flow rate of 80 mL/min, hydrogen and air were supplied to the FID. Fatty acids were identified by comparison of retention times with authentic standards.

To analyze the contents of TG, DG, MG and FFA after the hydrolysis reactions, the reaction products were separated by thin-layer chromatography (TLC) on a silica gel plate 5721 (Merck, Darmstadt, Germany) with $CHCl_3$ and acetone (96:4 vol/vol). Spots were measured with a dual-wavelength chromato scanner CS-930 (Shimadzu, Tokyo, Japan) at 465 nm.

RESULTS AND DISCUSSION

The aim of this work was to concentrate DHA from the glycerides of fish oil by hydrolysis with microbial lipases. After hydrolysis (Fig. 1) FFA was removed from the reaction system. (This mixture, which contained TG, DG and MG without FFA, is called the "glyceride mixture" in this article.) The major fatty acid components of the glyceride mixtures were then analyzed (Fig. 2). The glyceride mixtures derived from hydrolysis with *Candida cylindracea* lipase contained DHA at about 30%, *i.e.,* three times more than the original fish oil. The DHA content in the glyceride mixture increased as hydrolysis progressed. When the hydrolysis was 70%, the DHA content in the glyceride mixture had increased to 30%. The EPA content increased with hydrolysis at first, but it decreased above 40% hydrolysis (Fig. 3). The final EPA content in the glyceride mixture was 70% less than in the original fish oil (Table 2). After hydrolysis with five other lipases, the contents of DHA, EPA and OA in the glyceride mixtures were almost the same as those in the original fish oil. Figure 4 shows hydrolysis with *Chromobacterium viscosum* lipase. The hydrolysis behavior for three acids (DHA, EPA and OA) with the other four lipases was almost the same as for *Chromobacterium viscosum* lipase (data not shown). The results indicate that *Candida cylindracea* lipase appears to be unique in its resistance to DHA.

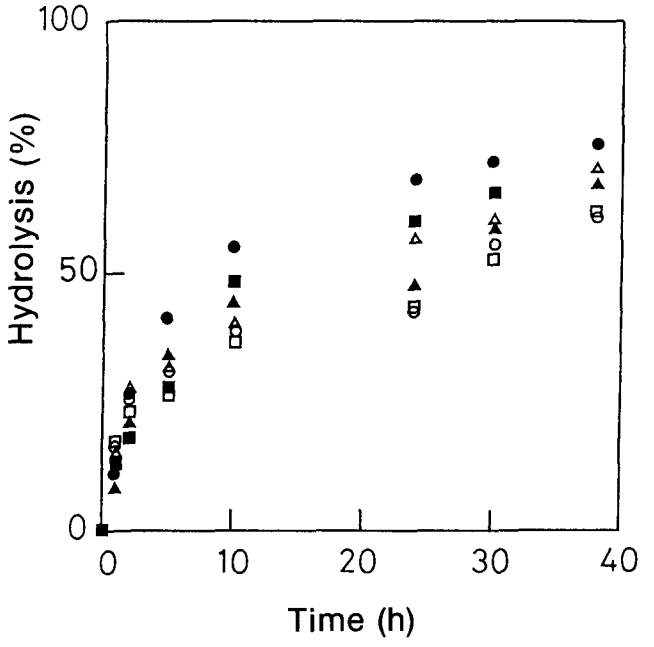

FIG. 1. Time courses of fish oil hydrolysis with microbial lipases. Amounts of lipases were 200 u/1 g oil. Reactions were run at 37°C, stirred at 500 rpm and bubbled with nitrogen gas. *Candida cylindracea* lipase (●), *Rhizopus delemar* lipase (○), *Aspergillus niger* lipase (▲), *Rhizopus javanicus* lipase (△), *Pseudomonas* sp. lipase (■) and *Chromobacterium viscosum* lipase (□).

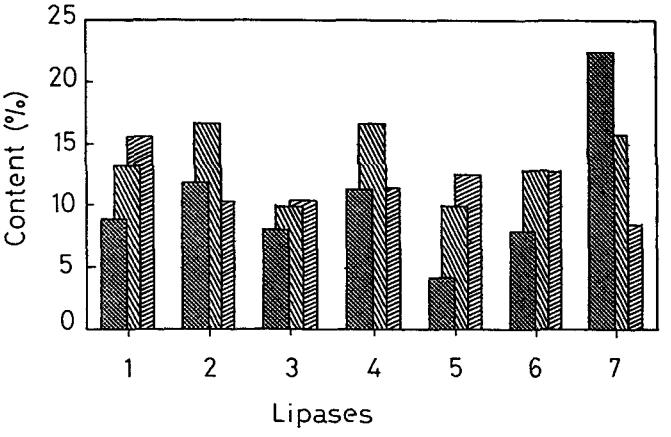

FIG. 2. Concentrations of docosahexaenoic acid (dotted bar), eicosapentaenoic acid (upward slanted bar) and oleic acid (downward slanted bar) in glyceride mixtures of fish oil that were hydrolyzed with lipases. Hydrolysis was 60%. Lipases: 1, original fish oil; 2, *Aspergillus niger* lipase; 3, *Rhizopus delemar* lipase; 4, *Rhizopus javanicus* lipase; 5, *Pseudomonas* sp. lipase; 6, *Chromobacterium viscosum* lipase; and 7, *Candida cylindracea* lipase.

Generally, water-soluble substrates (*e.g.,* proteins) in enzymatic reactions, the factors that determine the rate of reaction are enzyme concentration, substrate concentration, temperature, pH and metallic ions. However, in heterogeneous reactions, such as hydrolysis of oil, the water-oil ratio, stirring method, shape of reactor and the presence of surfactants, have a larger effect on the rate of reaction and formation of emulsions. The emulsion conditions

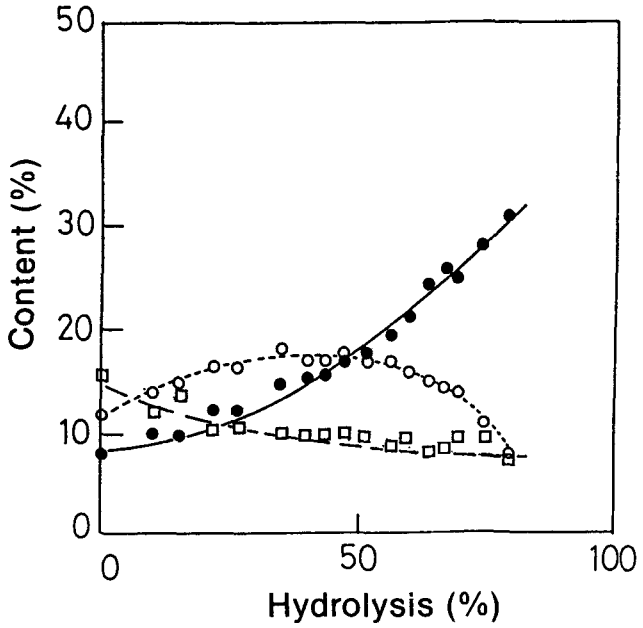

FIG. 3. Concentrations of docosahexaenoic acid (●), eicosapentaenoic acid (○) and oleic acid (□) in glyceride mixtures of fish oil that were hydrolyzed with *Candida cylindracea* lipase.

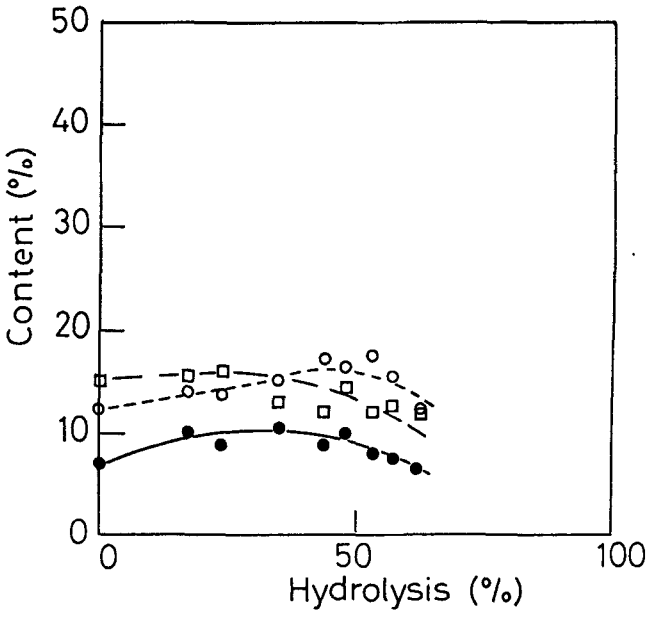

FIG. 4. Concentrations of docosahexaenoic acid (●), eicosapentaenoic acid (○) and oleic acid (□) in glyceride mixtures of fish oil that were hydrolyzed with *Chromobacterium viscosum* lipase.

TABLE 2

Major Fatty Acid Components of Original Fish Oil and the Glyceride Mixture[a]

	14:0	16:0	18:0	18:1	18:2	EPA	DHA
Fish oil	6.8	16.2	2.8	15.6	0.4	13.3	8.9
G.M.[b]	3.6	6.8	5.2	9.3	1.0	9.8	30.5

[a]EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[b]The glyceride mixtures (G.M.) were purified by the following method: (i) fish oil was hydrolyzed with *Candida cylindracea* lipase; (ii) when hydrolysis was 78%, 2 mL of reaction system was added to 10 mL of acetone and 10 mL of hexane; (iii) this reaction system was titrated with 1/2 N KOH-methanol solution and (iv) after titration, the reaction system was shaken and separated, the upper layer was evaporated and esterified with BF_3-methanol.

determine the rate of reaction. The reaction time depends on these factors and it is difficult to adequately estimate the specificities of lipases. In our work, however, the hydrolysis resistant value (HRV) was calculated, and the relationship between HRV and hydrolysis discounts the reaction time factor. To give an example, when the water-oil ratio was changed from 1:1 to 1:2, 1:5 and 1:10, these changes affected the rate of reaction, but the relationship between HRV and hydrolysis was the same as that obtained under the original conditions. HRV was calculated according to the following formula:

$$\text{HRV (\%)} = \{(100 \times GCa - B \times GGCb)/(100 \times GCa)\} \times 100$$

where GC*a* is the content of each fatty acid in the original oil measured by gas chromatography (%), GC*b* is the content of each fatty acid in FFA measured by gas chromatography (%) and B is the ratio of FFA in the reacted oil as measured by the chromato scanner (%).

The difference in HRV decrease with hydrolysis indicates the difference of lipase resistance to fatty acids. The HRV for hydrolysis of fish oil with *Candida cylindracea* lipase was significantly different from the other five lipases. In the case of hydrolysis with *Candida cylindracea* lipase, HRV patterns for DHA and EPA were different from those for saturated and monoenoic acids (Fig. 5). The other five lipases were slightly resistant to

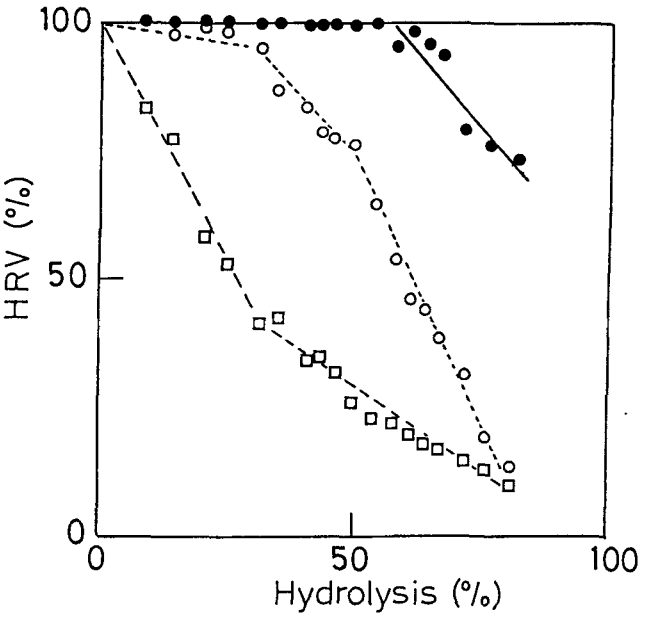

FIG. 5. Relationship between hydrolysis and hydrolysis resistance value (HRV). Fish oil was hydrolyzed with *Candida cylindracea* lipase. Docosahexaenoic acid (●), eicosapentaenoic acid (○) and oleic acid (□).

PUFA, and the difference of HRV patterns between DHA, EPA and OA were less than that for *Candida cylindracea* lipase (Fig. 6). As a result, the contents of DHA, EPA and OA were the same as in the original fish oil.

It has been reported that because n-3 PUFA is concentrated in the 2-position of TG, hydrolysis of fish oil with 1,3-specific lipase should produce PUFA-rich 2-MG and 1(3),2-DG (6). In this report, nonspecific and 1,3-specific lipases were tested, and nonspecific *Candida cylindracea* lipase was indeed most resistant to DHA. This result is due to acyl chain specificity, not to positional specificity. The other five lipases hydrolyzed PUFA to the same extent as saturated and monoenoic acids because they have less acyl chain specificity, and PUFA transfer from the 2- to the 1,3-position of TG.

To produce a higher concentration of DHA in the glyceride mixture, DHA-rich tuna oil was hydrolyzed with *Candida cylindracea.* The DHA content in the glyceride mixture increased with hydrolysis (Fig. 7). When hydrolysis was 65%, the DHA content was 53.1% (Table 3). This was twice that in the original tuna oil. The HRV pattern of hydrolyzed tuna oil showed the same tendency as that of the hydrolyzed fish oil (Fig. 8).

After 70% hydrolysis of tuna oil, the DHA contents in TG and DG were 52.4% (in TG) and 55.3% (in DG), respectively. This result suggests that there was TG in tuna oil that had two molecules of DHA. Because the DHA content in DG was about 50%, the DG was probably derived from hydrolyzed TG containing one or two molecules of DHA.

It had been presumed that because lipases are resistant to DHA in fish oil, the TG would decrease and DHA-rich DG and MG would increase in reaction mixtures at high-percentage hydrolysis. Actually, at 70% hydrolysis of tuna oil, the contents of TG, DG and MG in the glyceride

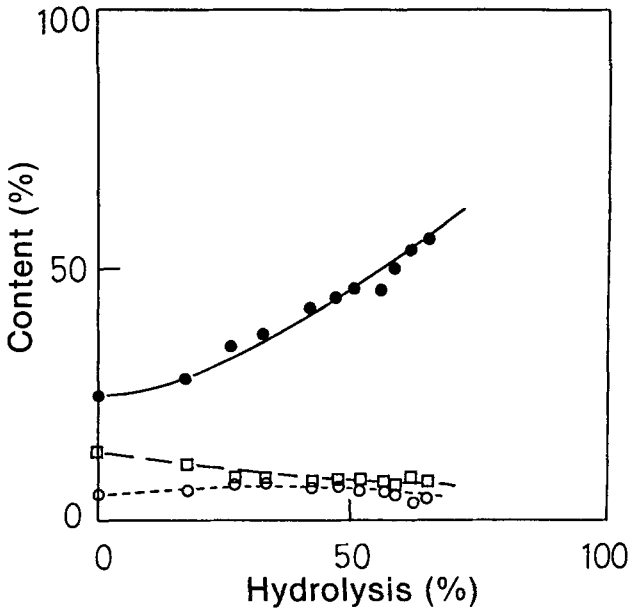

FIG. 7. Concentrations of docosahexaenoic (●), eicosapentaenoic acid (○) and oleic acid (□) in glyceride mixtures of tuna oil hydrolyzed with *Candida cylindracea* lipase.

TABLE 3

Major Fatty Acid Components of Original Tuna Oil and the Hydrolyzed (65%) Glyceride Mixture[a]

	14:0	16:0	18:0	18:1	18:2	EPA	DHA
Tuna oil	4.8	21.6	4.8	14.1	1.5	5.6	25.1
G.M.[b]	1.7	10.4	3.2	9.5	0.7	4.1	53.1

[a]As in Table 2. [b]As in Table 2.

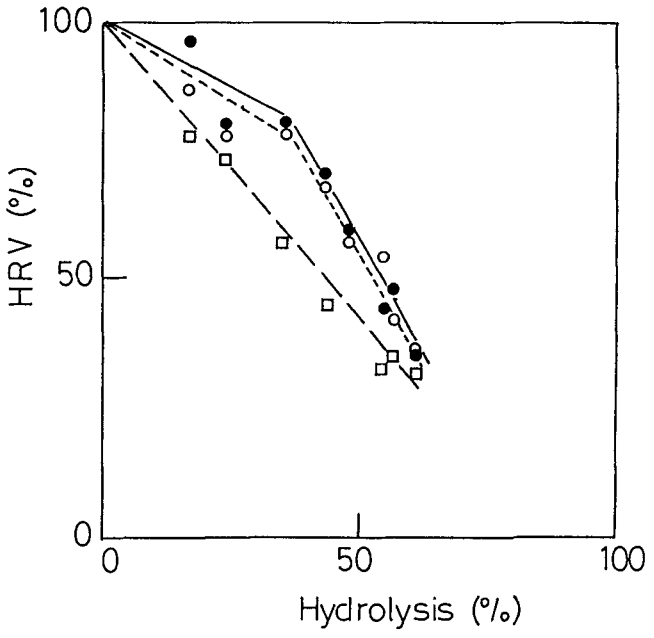

FIG. 6. Relationship between hydrolysis and hydrolysis resistance value (HRV). Fish oil was hydrolyzed with *Chromobacterium viscosum* lipase. Docosahexaenoic acid (●), eicosapentaenoic acid (○) and oleic acid (□).

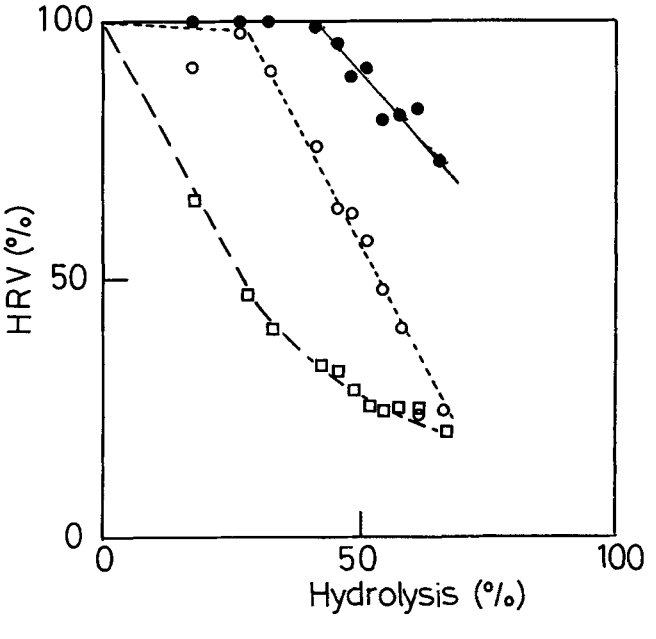

FIG. 8. Relationship between hydrolysis and hydrolysis resistance value (HRV). Tuna oil was hydrolyzed with *Candida cylindracea* lipase. Docosahexaenoic acid (●), eicosapentaenoic acid (○) and oleic acid (□).

mixture were 75% (TG), 24% (DG) and 1% (MG). These values were higher than expected. The reason for this is probably that the saturated and monoenoic acids in TG that did not contain DHA were more easily hydrolyzed with *Candida cylindracea* lipase than those in TG that did contain DHA. As a result, the TG levels in the glyceride mixtures stayed high.

Apparently, the *Candida cylindracea* lipase reaction takes place in two steps. In the first step, TG not containing DHA is hydrolyzed, and in the second step, TG containing DHA is hydrolyzed. The results suggest that *Candida cylindracea* lipase recognizes the whole TG molecular structure, not only its ester bonds.

REFERENCES

1. Anderson, R.E., *Exp. Eye Res. 10*:339 (1970).
2. Svennerholm, L.J., *Lipid Res. 9*:570 (1968).
3. Lawson, L.D., and B.G. Hughes, *Biochem. Biophys. Res. Commun. 152*:328 (1988).
4. Brockerhoff, H., *Biochim. Biophys. Acta 212*:92 (1970).
5. Alford, J.A., D.A. Pierce and F.G. Suggs, *J. Lipid Res. 5*:390 (1964).
6. Bottino, N.R., G.A. Vandenburg and R. Reiser, *Lipids 2*:489 (1967).
7. Suzuki, S. (ed.), Japanese Industrial Standards Committee, *Japanese Industrial Standard*, Japanese Standard Association, Tokyo, 1988, Method JIS K601.

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