Selective Hydrolysis of Fish Oil by Lipase to Concentrate n-3 Polyunsaturated Fatty Acids[†]

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Six lipases were examined for concentrating the n-3 polyunsaturated fatty acid (n-3 PUFA) of two kinds of fish oil (cod liver oil and refined sardine oil). Although all lipases could increase the n-3 PUFA content of the remaining glycerides, *Candida cylindracea* and *Aspergillus niger* lipases gave glycerides with a more than two-fold increase in n-3 PUFA content over the original fish oils.

Candida cylindracea lipase seems the most promising with respect to recovery of triacylglycerol. *Aspergillus niger* lipase increased not only the docosahexaenoic acid (DHA) content but also eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA), although the absolute value of the latter was quite low. The effects of temperature (15–40 °C) on the concentration of n-3 PUFA were investigated. Lower temperatures did not improve the concentration of n-3 PUFA, but prevented the development of an unpleasant odor in the product.

Fish oils such as those obtained from sardine, cod, mackerel, and squid contain 22–24% n-3 polyunsaturated fatty acids (n-3 PUFA).[1] n-3 PUFA such as eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) have pharmacological effects in relation to heart disease[2] and other related diseases. At present, n-3 PUFA ethyl ester is being sold as a physiologically functional oil. It was reported, however, that the triacylglycerol (TG) form was absorbed more easily than the ethyl ester during human digestion.[3] Therefore, it is desirable to produce TG which has a higher n-3 PUFA content.

n-3 PUFA is usually purified by chemical and physical methods such as urea complex-ation,[4] Corey's methods,[5,6] and HPLC.[7] In this saponification process, however, n-3 PUFA might be easily oxidized.

Another method was found recently using fungus cultivated in media containing fish oil or fish meal. By this method, n-3 PUFA is found to be concentrated in the undigested part of the fish oil.[8–10]

Concentration of n-3 PUFA in fish oil is also possible by enzymic treatments. As described in Japanese patents, when fish oil is partially hydrolyzed by a microbial lipase produced by *Candida cylindracea*,[11] *Alkaligenes* sp.,[12] or *Arthrobacter ureafaciens*,[13] the content of n-3 PUFA in the remaining glycerides is increased significantly. Also, TG highly en-

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riched with EPA or DHA have been prepared *via* interesterification of cod liver oil (CLO) and free fatty acid or ethyl ester of EPA or DHA, catalyzed by an immobilyzed 1,3-specific lipase from *Mucor miehei.*[14]

The objective of this study is to study the characteristics of lipase-catalyzed hydrolysis of fish oil, with the hope of obtaining oil having a higher n-3 PUFA content. Hydrolyses at lower temperatures were especially investigated to avoid possible nonenzymatic acyl migration, and also to suppress development of an unpleasant smell which is specific to fish oils.

Materials and Methods

Fish oils and enzymes. CLO was obtained from Peter Möller a/s (Oslo, Norway) with the following properties: acid value (AV), 0.33; saponification value (SV), 179.4; TG content, 99.8%; n-3 PUFA content, 22.02%. Refined sardine oil (RSO) was obtained from Nippon Oils and Fats Co., Ltd., Ohji Works (Tokyo, Japan): AV, 0.41; SV, 182.6; TG content, 99.04%; n-3 PUFA content, 28.59%.

Six types of lipases were obtained from different sources as shown in Table I. Lipases 1, 3, 4, 6, and 7 have 1,3-positional specificity, while the others do not have this characteristic. Lipase 1 is produced using genetic engineering techniques (the lipase gene was cloned from *Humicola lanuginosa* and its lipase was produced by transformed *Aspergillus oryzae*).

Hydrolysis reaction. The required amounts of lipase powder were dissolved in 0.1 M phosphate buffer to prepare the following concentrations: 2,000, 5,000 and 10,000 U/6 ml enzyme solution. A unit of enzyme was defined by the Japanese industrial standard method (A).[15] The reaction mixture contained 6 ml of the enzyme so-

lution and 4 ml of the fish oil amounting to 10 ml of reaction mixture. It was agitated in a flat-bottomed bottle (32 mm i.d. and 100 mm height) made of glass, with a magnetic stirrer bar at 700 rpm. Air in the bottle was replaced by nitrogen gas. In some cases, the reaction mixture became semi-solid so that the stirring bar could not rotate, but incubation of the semi-solid reaction mixture was continued. Most reactions were done at 20°C for 100 hr. After the reaction, fish oil was extracted by the Folch method.[16]

Analytical procedures. The AV[17] of the extracted fish oil was analyzed and hydrolysis (%) was calculated using an equation from our previous report.[18] Lipid composition (TG, 1,3-DG, 1,2-DG, MG, FFA) was analyzed by a TLC/FID analyzer (Iatroscan TH-10, Yatoron Laboratories, Inc., Tokyo, Japan). The chromarods were developed in a solvent composed of benzene–chloroform–acetic acid = 70 : 30 : 2, (v/v). To remove FFA from the extracted fish oil, 0.2 ml of the extracted fish oil was dissolved in 10 ml of *n*-hexane. Then 10 ml of methanol and 10 ml of distilled water were added. This mixture was titrated with methanolic 0.5 N KOH solution to shift the pH of the mixture to alkaline. The mixture was separated into two phases by centrifugation. The upper layer containing neutral glycerides was taken out to evaporate the solvent. The remaining oil was separated into the neutral glycerides (TG, DG, and MG fractions) by thin layer chromatography (TLC, Kieselgel 60 F253, Merck). TLC plates were developed in a solvent composed of *n*-hexane–ether–acetic acid = 70 : 30 : 1, (v/v). The three samples, *i.e.*, the neutral glycerides and the TG and DG fractions, were methylated using the method of Prevot and Mordret.[19] Fatty acid methyl esters were analyzed by capillary gas chromatography. Gas chromatographic analysis conditions were as follows: column, 50 m × 0.25 mm i.d., 0.2 μm fused silica WCOT CP-Sil88 (Chrompack, Holland); column temperature, 160–220°C, 4°C/min; carrier gas, N_2, 200 kPa, 28 cm/sec; injector, splitter, $T = 250°C$; detector, FID (Hitachi Ltd., Tokyo, Japan), $T = 250°C$.

Table I. Lipases Examined

|	Enzyme origin	Abbreviations	Manufacturer	Activity (U/mg powder)	Reaction pH
1.	*Humicola lanuginosa* (*Aspergillus oryzae*)	FL	Novo Industri a/s	2290	8.0
2.	*Candida cylindracea*	CC	Meito Sangyo Co., Ltd.	246	7.0
3.	*Aspergillus niger*	AN	Amano Pharmaceutical Manufacturing Co., Ltd.	20.1	7.0
4.	*Rhizopus delemar*	RD	Amano Pharmaceutical Manufacturing Co., Ltd.	16.1	7.0
5.	*Geotrichum candidum*	GC	Amano Pharmaceutical Manufacturing Co., Ltd.	32.5	7.0
6.	Porcine pancreas (crude)	PP	Sigma Chemical Co.	80.4	7.5
7.	Porcine pancreas (pure)	PP	Sigma Chemical Co.	2370	7.5
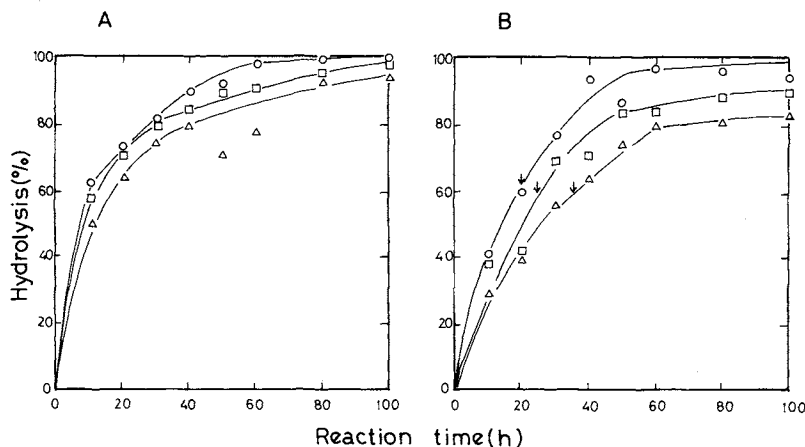
Fig. 1. Effects of Lipase Concentration on the Hydrolysis of Cod Liver Oil.

A, CC lipase; B, AN lipase. The amount of enzyme used was: △, 2,000 U; □, 5,000 U; ○, 10,000 U. Reaction temperature was 20°C. Marks ↓ indicate the times when the reaction mixture became semi-solid.

Results and Discussion

Courses of hydrolysis of fish oil by lipase

Considering CC and AN lipases to be representative of positionally nonspecific and 1,3-positinally specific lipases respectively, the courses of hydrolysis of fish oil by these have been compared. Figure 1 shows the courses of hydrolysis of CLO by CC(A) and AN(B) lipases. HL lipase gave a somewhat similar profile to AN lipase (data not shown). CC lipase gave a higher degree of hydrolysis than AN lipase at the same number of enzyme units used. Interestingly, the reaction mixture hydrolyzed by AN lipase became solid at the times shown by the vertical arrows, but those hydrolyzed by CC lipase did not throughout the reaction time. The change of state from liquid to semi-solid was probably due to a preferential crystallization of a greater amount of saturated fatty acids, the melting points of which are higher than those of unsaturated fatty acids. It is seen from Fig. 1 that the hydrolysis percentage was not proportional to the enzyme concentration even at early stages of the reaction. This seems, however, to be usual in heterogeneous enzymic reactions as reported by one of us previously.[15] Hydrolysis profiles by various lipases when enzyme units were the same are shown in Fig. 2.
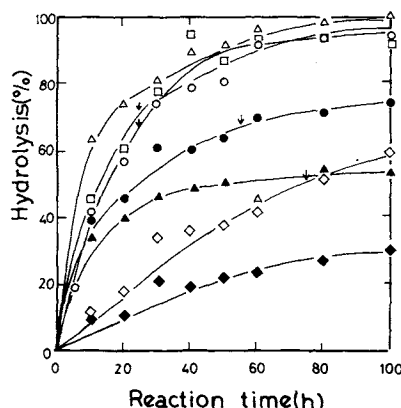

Fig. 2. Profiles of Hydrolysis of Cod Liver Oil by Various Lipases.

○, *Humicola lanuginosa* (*Aspergillus oryzae*) lipase; △, *Candida cylindracea* lipase; □, *Aspergillus niger* lipase; ●, *Rhizopus delemer* lipase; ▲, *Geotrichum candidum* lipase; ◇, porcine pancreas lipase (crude); ◆, porcine pancreas lipase (pure). The amount of enzyme used was 10,000 U and the reaction temperature was 20°C. Marks ↓ show the times at which the reaction mixture became semi-solid.

There is no clear correlation between the positional specificity and the ease (rapidity) of the fish oil hydrolysis. This implies that the concept of positional specificity alone cannot explain the observed diversity in the hydrolysis of the fish oil. Iwai and Tsujisaka pointed out that the course of oil hydrolysis by lipases is decided by various factors such as 1) differ-

ences in substrate specificities including fatty acid and positional specifities; 2) differences in the rate of the reverse reaction which occurs during the course of oil hydrolysis by lipases; 3) differences in the reactivity of each lipase toward partial glycerides.[20] Therefore, to distinguish between these effects during fish oil hydrolysis, further experiments using mono-acid TGs (TG whose three fatty acid residues have the same chemical structure) such as TGs of EPA only or DHA only should be done.

The composition of oil obtained by the lipase-catalyzed hydrolysis of CLO is shown in Fig. 3. It was found that even in the early stages of hydrolysis, 1,3-DG was either not detected or present at very low concentrations. It is also noticeable that the major glyceride fraction (unhydrolyzed by the lipases) detected almost throughout the reaction was TG. The content of MG was always small compared to TG and DG.
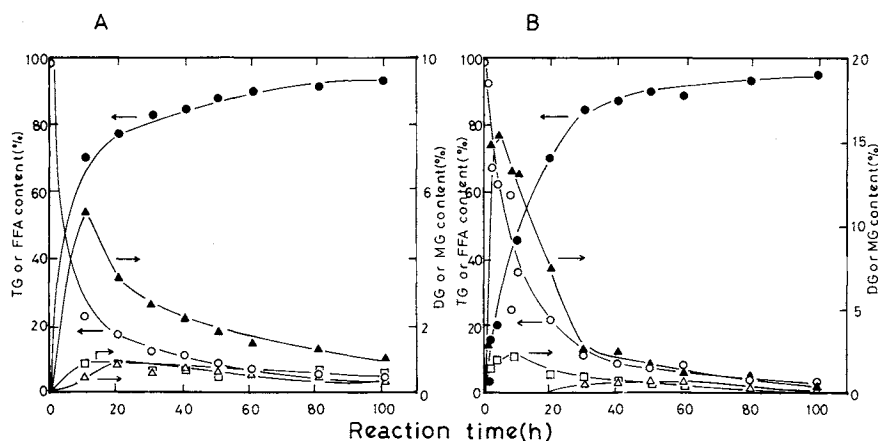

Fig. 3. Changes in the Composition of Lipid Components Obtained by Lipase-catalyzed Hydrolysis of Cod Liver Oil.

The amount of enzyme used was 10,000 U and the reaction temperature was 20°C. A, CC lipase; B, AN lipase. ○, TG; ●, FFA; △, 1,3-DG; ▲, 1,2-DG; □, MG.
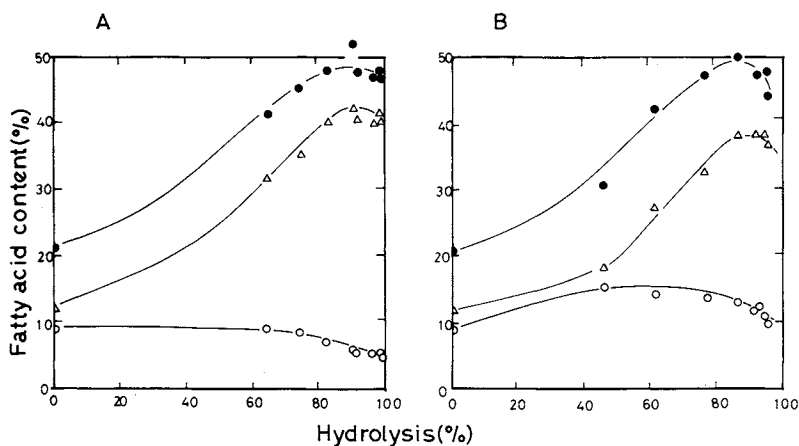

Fig. 4. Changes in n-3 PUFA Content of FFA-Free Glycerides Obtained by Lipase-catalyzed Hydrolysis of Cod Liver Oil.

The amount of enzyme used was 10,000 U and the reaction temperature was 20°C. A, CC lipase; B, AN lipase. ○, EPA; △, DHA; ●, total n-3 PUFA.

n-3 PUFA content of FFA-free glycerides obtained by lipase treatment

Samples of FFA-free glycerides extracted from CLO hydrolyzed by lipase were analysed for n-3 PUFA content. The results, expressed as n-3 PUFA content *versus* percent of hydrolysis, are shown in Fig. 4. CC and AN lipases gave significant increases in n-3 PUFA contents as the hydrolysis progressed. CC lipase concentrated only DHA with little increase in EPA, while AN lipase concentrated DHA with partial concentration of EPA. However, extensive hydrolysis approaching 100% resulted in a decrease in EPA content for both lipases. Funada and Tanaka also reported recently that EPA content was increased considerably but the DHA content was increased little by CC lipase.[21] Maximal total n-3 PUFA contents were about 50% for both lipases. Other lipases were less effective in increasing the n-3 PUFA contents. HL, RD, GC and PP lipases gave maximal total n-3 PUFA contents of 40%, 38%, 35% and 32%, respectively. All four lipases increased DHA contents more or less, but increased EPA contents very little. This clearly demonstrates that the EPA ester bond can be hydrolyzed more easily by lipase than the DHA ester bond. DHA has a double bond closer to the carboxyl group (at the 4-position) than EPA

(5-position). Miller *et al.* have recently reported from their study on the effects of unsaturation on the esterification rate by immobilized *Mucor miehei* lipase that the reaction rate was higher when the first double bond in the substrate was in the 3-position as opposed to the 2-position.[22] Therefore our result supports their finding, although our reaction (hydrolysis) is the reverse.

Since CC and AN lipases gave promising results, we then did the same lipase-catalyzed hydrolysis using RSO as substrate, the results of which are depicted in Fig. 5. The profiles of EPA, DHA and total n-3 PUFA contents were similar to those in Fig. 4 (CLO) although RSO contained a higher concentration of n-3 PUFA (about 28%) than CLO (about 22%). To get a better insight of the n-3 PUFA distribution of each glyceride, TG and DG of enzyme-treated glyceride were separated by TLC (the MG content was so small that its analysis was omitted), and the EPA, DPA, and DHA contents were measured. The results for TG, summarized in Fig. 6 A–D, show the changes in individual n-3 PUFA content during incubation together with the hydrolysis (%) and total n-3 PUFA content of TG. Only the DHA content was increased significantly. The DPA content was changed a little but much less than those of EPA and DHA. The rate of increase
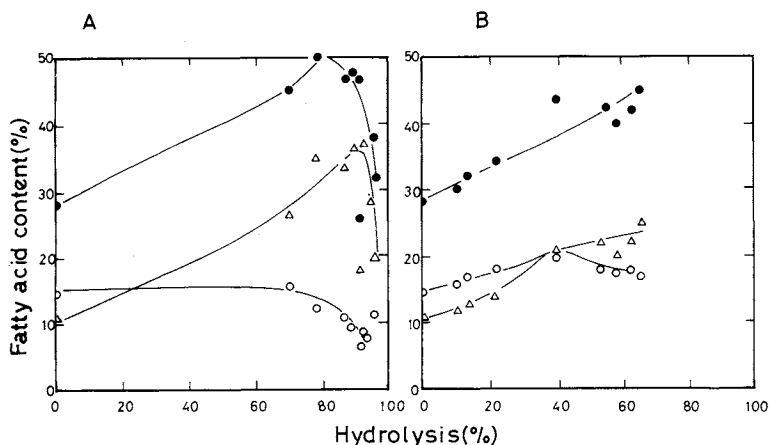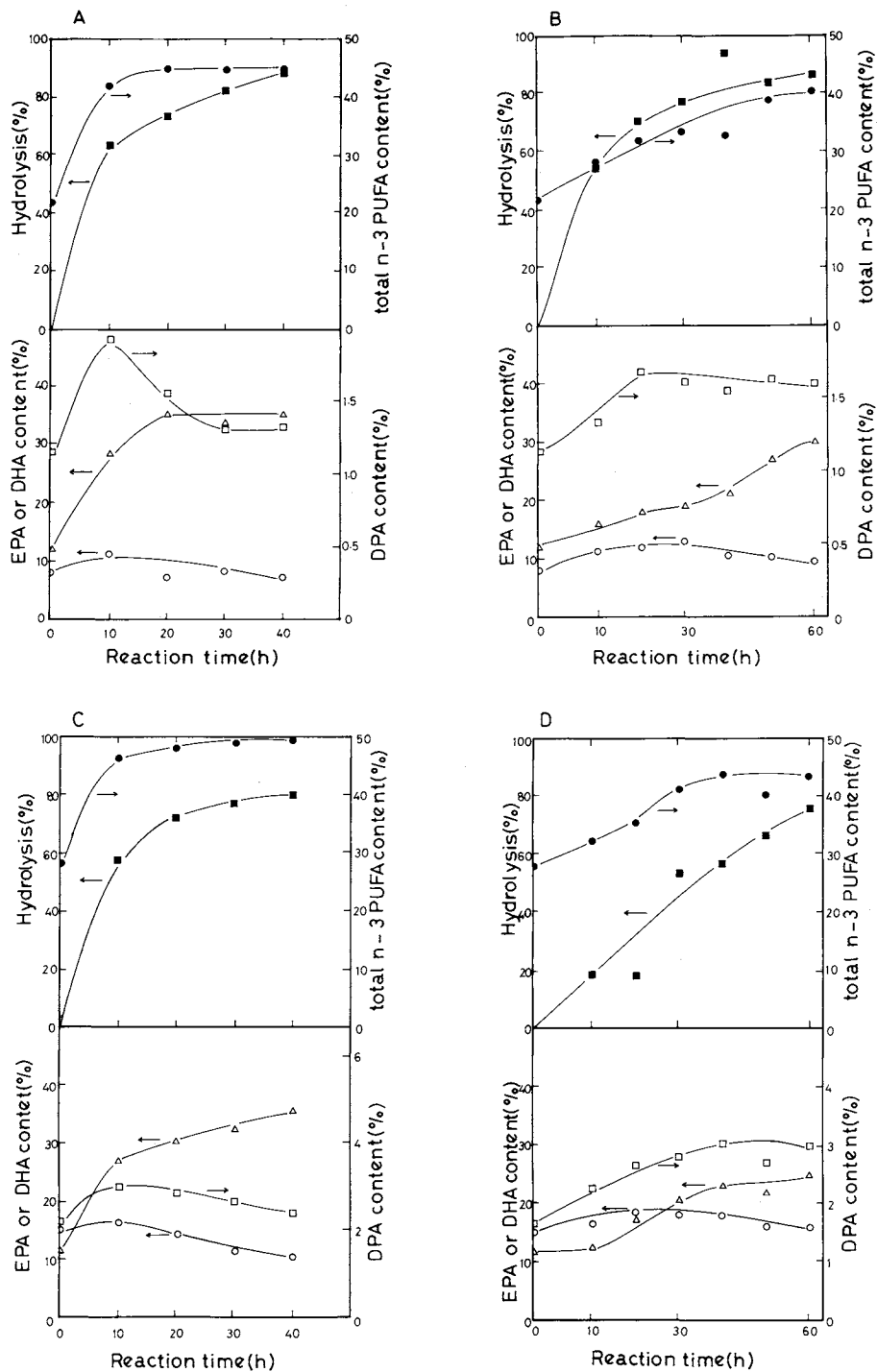

Fig. 5. Changes in n-3 PUFA Content of FFA-Free Glycerides Obtained by Lipase-catalyzed Hydrolysis of Refined Sardine Oil.

The amount of lipase used was 10,000 U and the reaction tempeature was 20°C. A, CC lipase; B, AN lipase. ○, EPA; △, DHA; ●, total n-3 PUFA.

Fig. 6. Changes n-3 PUFA Content of TG Fraction Remaining during Lipase-catalyzed Hydrolysis of Fish Oil.

Profiles of hydrolysis (%) are also depicted. The amount of lipase used was 10,000 U and the reaction temperature was 20°C. A, cod liver oil-CC lipase; B, cod liver oil-AN lipase; C, refined sardine oil-CC lipase; D, refined sardine oil-AN lipase. ○, EPA; △, DHA; □, DPA; ●, total n-3 PUFA; ■, hydrolysis (%).

of n-3 PUFA and its final concentration were higher for CC lipase hydrolysis than for AN lipase hydrolysis.

Although data are not shown, the EPA content of TG was higher than that of DG but the DHA content of DG was higher than that of TG, and total n-3 PUFA contents of DG were greater than those of TG, but the differences (5–13%) were not significant. Since the DG content of enzyme-hydrolyzed oil is smaller when hydrolysis is greater than 50% (Fig. 3), use of the enzyme-treated oil excluding DG and MG as well as FFA is admissible without significant loss of n-3 PUFA. It is noteworthy that the n-3 PUFA content of TG was more than 40% at a relatively low level of hydrolysis (about 40%) when either fish oil was hydrolyzed by CC lipase, but for AN lipase, a considerably higher level of hydrolysis was required to achieve a comparable n-3 PUFA concentration.

Effects of temperature on n-3 PUFA concentration

In this study, a lower reaction temperature was used in order to minimize the possibility of nonenzymatic acyl migration. To compare n-3 PUFA contents at various reaction tempera-
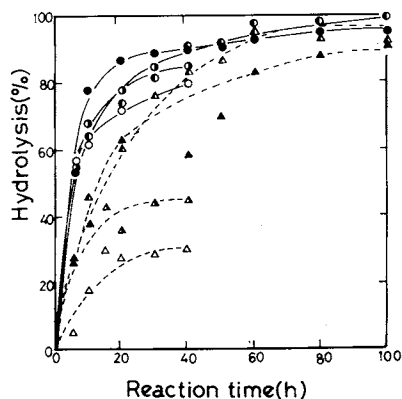
tures, hydrolyses were done at 15, 20, 30, and 40°C, and Fig. 7 shows the courses of hydrolysis of CLO by CC and AN lipases. The use of higher temperatures did not cause a higher final hydrolysis percentage, probably because of heat inactivation of the enzymes, and AN lipase appeared to be less stable thermally than CC lipase. Figure 8 shows the composition of CLO hydrolyzed by CC lipase at 15, 30, and 40°C. As in the case of hydrolysis at 20°C (Fig. 3), the major glyceride was TG even at a high hydrolysis percentage. At higher temperatures (30 and 40°C), the contents of 1,3-DG and 1,2-DG became reversed during incubation, which might be. due to non-enzymatic acyl migration. The effect of acyl migration was not significant at moderate

Fig. 7. Effects of Temperature on the Hydrolysis of Cod Liver Oil by the Two Lipases.

The amount of lipase used was 10,000 U. —●—, at 15°C by CC lipase; —◐—, at 20°C by CC lipase; —◑—, at 30°C by CC lipase; —○—, at 40°C by CC lipase; ---▲---, at 15°C by AN lipase; ---▲---, at 20°C by AN lipase; ---▲---, at 30°C by AN lipase; ---△---, at 40°C by AN lipase.
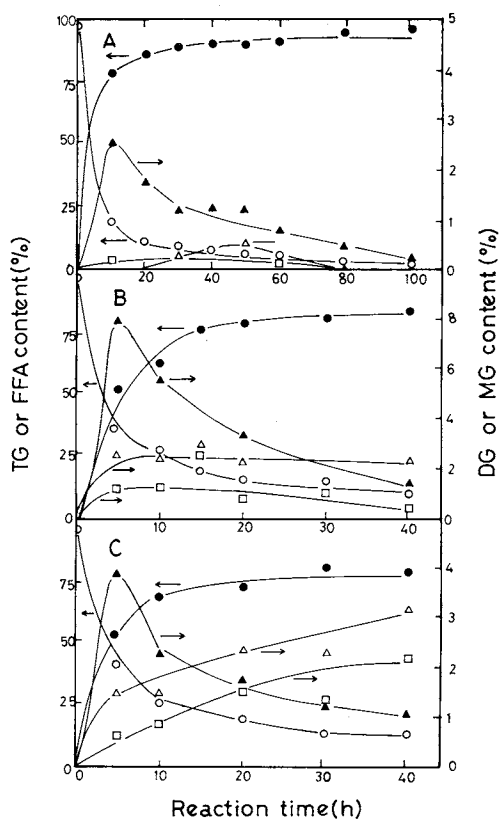

Fig. 8. Effects of Temperature on the Composition of the Lipid Obtained from Cod Liver Oil Hydrolyzed by CC Lipase.

The amount of lipase used was 10,000 U. A, 15°C; B, 30°C; C, 40°C. ○, TG; ●, FFA; △, 1,3-DG; ▲, 1,2-DG; □, MG.

Table II. RECOVERIES OF GLYCERIDES AND n-3 PUFA AT 20°C (ENZYME USED = 10,000 U)

Fish oil	Enzyme	Reaction time (hr)	n-3 PUFA content (%)		Recoveries of glycerides (%)		Recoveries of n-3 PUFA (%)	
			TG	Glycerides	TG	Glycerides	TG	Glycerides
CLO	CC	10	42.0	43.1	22.7	29.4	51.3	55.3
		20	44.8	45.6	17.6	22.6	25.8	46.7
		30	43.7	48.7	12.7	16.5	17.9	36.5
		40	44.5	50.2	11.4	14.9	15.0	32.3
CLO	AN	10	28.6	31.4	66.1	79.2	85.7	92.1
		20	31.7	42.2	53.2	64.0	76.5	90.1
		30	33.3	47.7	42.7	52.3	64.7	78.9
		40	33.0	47.7	33.6	40.1	50.3	62.5
		50	38.8	49.1	23.9	29.3	42.3	59.3
		60	40.3	47.7	18.8	22.8	33.4	53.4
RSO	CC	10	46.4	46.3	39.2	53.9	26.8	47.9
		20	48.1	49.8	21.8	29.7	21.3	35.5
		30	48.8	50.5	11.1	14.8	11.7	22.3
		40	48.9	50.3	8.64	12.2	10.9	19.6
RSO	AN	10	32.2	36.4	75.5	85.3	85.2	96.2
		20	38.6	35.4	35.5	46.6	49.2	62.9
		30	41.6	43.1	29.2	37.9	42.2	55.1
		40	43.7	42.5	23.2	29.8	35.4	45.5
		50	40.4	48.9	24.7	30.5	36.0	47.0
		60	43.7	46.3	18.6	23.6	28.5	38.2

temperature (20°C) when a positionally specific lipase (AN) was used (see Fig. 3B). Suitable data could not be obtained at higher temperatures due to inactivation of AN lipase.

Although hydrolysis at lower temperatures did not result in a higher concentration of n-3 PUFA in residual glycerides, very little un-

pleasant odor developed compared to reactions at higher temperatures, even during long incubation periods.

Recoveries of glycerides and n-3 PUFA

The following four recoveries were defined and calculated.

$$\text{Recovery of TG (\%)} = \frac{\text{amount of TG remaining}}{\text{amount of fish oil used}} \times 100$$

$$\text{Recovery of glycerides (\%)} = \frac{\text{amount of (TG + DG + MG)}}{\text{amount of fish oil used}} \times 100$$

Recovery of n-3 PUFA in TG fraction only (%)

$$= \frac{\text{amount of TG remaining} \times \text{n-3 PUFA content of TG}}{\text{amount of fish oil used} \times \text{n-3 PUFA content}} \times 100$$

Recovery of n-3 PUFA in (TG + DG + MG) fraction (%)

$$= \frac{\text{amount of (TG + DG + MG) remaining} \times \text{n-3 PUFA content of (TG + DG + MG)}}{\text{amount of fish oil used} \times \text{n-3 PUFA content}} \times 100$$

The calculated data are summarized in Table II, and the following points are notable; 1) In case of CLO, when n-3 PUFA content in TG was higher than 40%, the recovery of TG was less than 25% and the recovery of n-3 PUFA was less than 50%.

2) Even longer incubation times of CLO with CC lipase did not yield an n-3 PUFA content in TG of more than 50% with a large decrease in the recovery of TG.

3) When RSO was hydrolyzed by CC lipase, a higher recovery of TG was obtained with a lower recovery of n-3 PUFA than in the hydrolysis of CLO by CC lipase.

4) Hydrolysis of RSO by AN lipase gave a better recovery of n-3 PUFA than by CC lipase.

General discussion

It is possible to obtain TG having an n-3 PUFA content of greater than 40% by a selective lipase-catalyzed hydrolysis followed by removal of FFA, DG, and MG (by extraction and molecular distillation). Of the six lipases evaluated in this study, CC lipase seems to be the best because the enzyme rapidly yields an oil having TG of the desired composition although the recovery of such TG is only 30–40%. To obtain a mixture of TG, DG, and MG having a high n-3 PUFA content, AN lipase is the most promising because the enzyme yields FFA-free glyceride having almost 50% n-3 PUFA content although the recovery of such glyceride is only 30%. Such glycerides may be converted to TG through an esterification reaction with n-3 PUFA (or its ester) by the action of another suitable lipase.

Among the six lipases tested, no lipase could raise the EPA content significantly.

As the recovery of n-3 PUFA-concentrated TG or glycerides is only 20–30%, exploitation of a more effective lipase is highly desirable. Further investigation is required in this area.

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