Production of n-3 Polyunsaturated Fatty Acid-Enriched Fish Oil by Lipase-Catalyzed Acidolysis Without Solvent[1]

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Fish oil rich in n-3 polyunsaturated fatty acid (n-3 PUFA) was prepared by nonsolvent enzymic acidolysis. n-3 PUFA-enriched fish oil contained 25% eicosapentaenoic acid (EPA) and 40% docosahexaenoic acid (DHA). In acidolysis of cod liver oil, EPA content of the original fish oil was reduced at 5 h, but DHA content of the fish oil increased. It was assumed that EPA in the fish oil was replaced by DHA to reach a new chemical equilibrium. Two-stage acidolysis, which was carried out under CO_2 replacement early (about 3 h) and also in vacuum at 5–24 h, was effective for reduction in the content of diacylglycerol, which was formed by reverse reaction, hydrolysis. This method has industrial significance because PUFA-enriched triacylglycerol is easily separated from the reaction mixture by molecular distillation.

KEY WORDS: Acidolysis, docosahexaenoic acid, eicosapentaenoic acid, immobilized lipase, lipase, n-3 polyunsaturated fatty acid.

Polyunsaturated fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have biochemical and pharmacological effects on human health. At present, n-3 PUFA-enriched fatty acid ethyl ester (PUFA-Et) is being produced as an essential oil. It was reported previously (1,2) that n-3 PUFA was most promptly absorbed from the intestines when free fatty acid (FFA) was given orally, moderately absorbed as triacylglycerol (TG) and poorly absorbed as PUFA-Et. However, free n-3 PUFA is oxidized most easily and, moreover, free n-3 PUFA is unacceptable as a food. Therefore, TG is considered to be the most desirable chemical form as a food.

Many researchers have carried out investigations on production of n-3 PUFA-enriched TG through selective hydrolysis (3), glycerolysis (4–6), interesterification (7) and acidolysis (4–6). In view of industrial downstream treatments, such as separation and purification of products (n-3 PUFA-enriched TG) from reaction mixtures (composed of TG, free n-3 PUFA or n-3 PUFA-Et and other byproducts), acidolysis seems most promising for separating the product and the substrate from these reaction mixtures. In this study, we elucidate the optimum conditions of acidolysis for preparation of n-3 PUFA-enriched TG.

EXPERIMENTAL PROCEDURES

Fish oil and enzyme. Cod liver oil (CLO), n-3 PUFA and n-3 PUFA methyl ester (n-3 PUFA-Me) were obtained from Peter Möller a/s (Oslo, Norway). n-3 PUFA contents of CLO and of the n-3 PUFA and n-3 PUFA-Me were 22.2% (8.6% EPA and 12.7% DHA) and 76.0% (31.6% EPA and 42.0% DHA), respectively. Refined sardine oil (RSO) was obtained from Nippon Oils and Fats Co., Ltd., Ohji Workes (Tokyo, Japan). RSO contained 28.6% n-3 PUFA (15.0% EPA and 11.4% DHA). Free EPA was obtained from Taiyo Fisheries Co., Ltd., Taiyo Central R & D Institute (Tsukuba, Japan), and its EPA content was 99.2%. Partially hydrolyzed CLO was prepared with *Candida cylindracea* lipase at 30°C for 30 min according to the method previously described (9). Its n-3 PUFA content was 32.0%, and its lipid composition was TG, 86.4%; and diacylglycerol (DG), 10.6%.

As lipase, immobilized *Mucor miehei* lipase (Lipozyme IM-60; Novo Nordisk Bioindustry Ltd., Bagsvaerd, Denmark) was used throughout this study. Its enzyme activity was 4.53×10^3 U/g [hydrolysis activity, one unit of enzyme is defined by the Japanese industrial standard, method A (8)].

Acidolysis reaction. Acidolysis reactions were carried out at 40°C for various periods. The reaction mixture contained various proportions of CLO (or RSO) and free n-3 PUFA (1:1–1:8, vol/vol/vol) and immobilized enzyme (0.25–1.00 g), amounting to a total of 5 mL reaction mixture. It was agitated at 600 rpm in a 10-mL flask with a magnetic stirrer bar. Air in the flask was replaced by CO_2 gas. The reaction conditions for interesterification were the same as for the acidolysis reaction, and the reaction mixture contained CLO and n-3 PUFA-Me instead of FFA.

Analytical procedures. Changes in lipid composition were analyzed by a TLC/FID (thin-layer chromatography/flame ionization detector) analyzer (Iatroscan MK-5, Yatoron Laboratories, Inc., Tokyo, Japan). The chromarods were developed in a solvent composed of benzene:chloroform:acetic acid (70:30:2, vol/vol/vol). To remove FFA from the sampled reaction mixture, 0.2 mL of the sample was dissolved in 10 mL of *n*-hexane. The 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 TG and DG, was taken out to evaporate the solvent. TG and n-3 PUFA-Me, as the interesterification reaction mixture, were separated by TLC (Kieselgel 60 F253; Merck, Darmstadt, Germany). TLC plates were developed in a solvent composed of *n*-hexane:ether:acetic acid (70:30:1, vol/vol). The TG fraction was methylated, and fatty acid methyl esters were analyzed by capillary gas chromatography. Details of these methods were reported previously (3).

Water content of the reaction mixture was determined by a Karl-Fischer moisture meter (MKS-1; Kyoto Electronics Corp., Kyoto, Japan).

RESULTS AND DISCUSSION

Linolenic acid incorporation in various oils. Model experiments, where linolenic acid was incorporated into CLO, partially hydrolyzed CLO or triolein, were carried

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out to elucidate characteristics of substrate and reaction. The incorporation rate of linolenic acid depended on the kind of substrate, but the final linolenic acid content in these oils was almost the same (data not shown). CLO had a lower incorporation rate than triolein, probably because CLO has more PUFA, and the microbial lipase has a lower specificity with PUFA (3,9). The highest incorporation rate was observed for the partially hydrolyzed CLO, because esterification as well as acidolysis took place.

Because n-3 PUFA is relatively expensive, lesser amounts of its use are preferable. The following composition was adopted throughout this study—TG: free n-3 PUFA—1:4 or 1:1 (vol/vol), and the amount of enzyme was 0.75 g/5 mL of the total reaction mixture.

Effects of various substrates on acidolysis. When pure, free EPA was used, EPA was incorporated up to 57% in CLO by acidolysis (Fig. 1A). However, when acidolysis was carried out with CLO and n-3 PUFA (EPA + DHA), the EPA content of CLO was reduced at 5 h, and the DHA content of TG increased (Fig. 1B). It was thought that EPA in CLO was replaced by DHA to reach a new chemical equilibrium because the total n-3 PUFA content in TG was unchanged.

When n-3 PUFA-Me was used, a higher content of total n-3 PUFA of TG was obtained (Fig. 1C). However, separation of unreacted methyl esters from the reaction mixture requires chromatography with large amounts of solvent(s), such as methanol and chloroform. Pure, free EPA is expensive. Hence, we focused further research on acidolysis because separation of FFA from the reaction mixture is feasible by molecular distillation.

Effects of CO_2 replacement and vacuum during acidolysis. The water content of the reaction mixture was supposed to be an important factor during acidolysis. Figure 2 (A and B) indicates time courses of the acidolysis reaction under CO_2 gas and in vacuum, respectively. During acidolysis carried out under CO_2 gas replacement, EPA incorporation in CLO increased rapidly at 0–2 h, yet DHA incorporation increased slowly; at 2–24 h, EPA content of CLO decreased a little and DHA content of CLO increased; finally, DHA content of CLO was higher than that of EPA. This is probably because the DHA ester bond is more resistant to hydrolysis of lipase than the EPA ester bond. Enzyme-modified CLO, prepared by acidolysis under CO_2 replacement, contained about 65% n-3 PUFA (40% DHA and 25% EPA). The same result was obtained from RSO (data not shown). When the reaction was carried out under CO_2, however, TG content decreased (Fig. 3A). When the enzymic reaction was carried out in vacuum, less DG was produced and the TG content was not reduced (Fig. 3B). However, n-3 PUFA incorporation of CLO was stopped at about 2 h; hence, the DHA content of CLO did not increase (Fig. 2B).

Table 1 shows changes in the free water content of the reaction mixture during acidolysis. The water contents of reaction mixtures increased at the beginning of the reaction, both under CO_2 gas replacement and in vacuum. Certainly, the immobilized enzyme particles contained a little water, which dissolved into the reaction mixture. Under CO_2 gas replacement, the free water content in the reaction mixture increased. In vacuum, however, the free water content was reduced. This was why the reaction stopped early when vacuum was used. The water content of the reaction mixture probably was reduced to less than

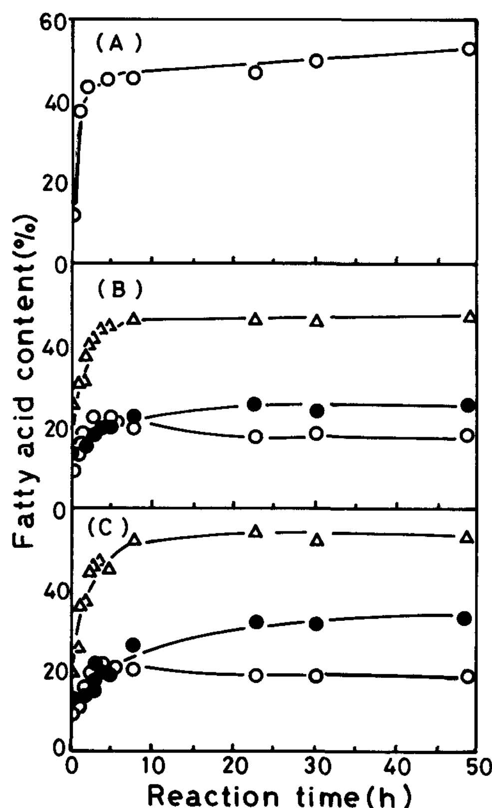

FIG. 1. n-3 Polyunsaturated fatty acid (PUFA) incorporation in cod liver oil by various reactions. Unlyophilized immobilized enzyme was used. Cod liver oil, substrate (1:1). A, eicosapentaenoic acid (EPA); B, n-3 PUFA; and C, n-3 PUFA-Me. ○, EPA; ●, docosahexaenoic acid; △, n-3 PUFA.

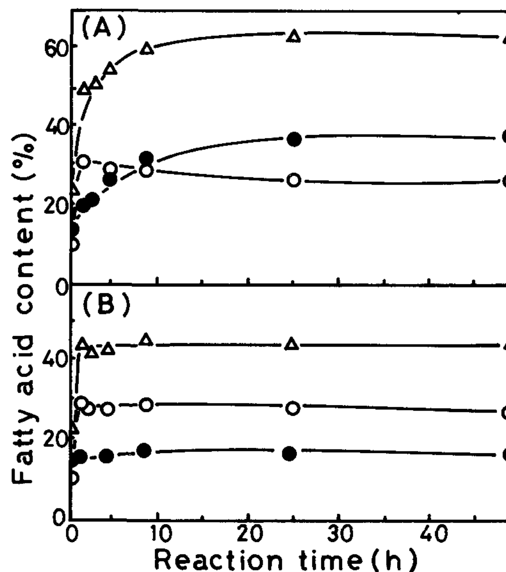

FIG. 2. Acidolysis of cod liver oil with n-3 polyunsaturated fatty acid (PUFA) by lipase. Unlyophilized immobilized enzyme was used. Cod liver oil, n-3 PUFA (1:4). A, with CO_2 replacement; B, in vacuum. ○, Eicosapentaenoic acid; ●, docosahexaenoic acid; △, n-3 PUFA.

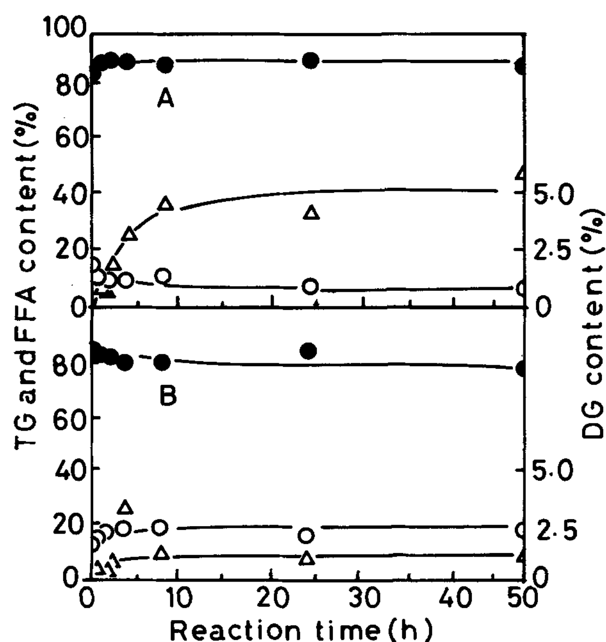

FIG. 3. Changes in the lipid components during acidolysis of cod liver oil with n-3 polyunsaturated fatty acid (PUFA) by lipase. Cod liver oil, n-3 PUFA (1:4). A, with CO_2 replacement; B, in vacuum. ○, Triglyceride; ●, free fatty acid; △, diglyceride.

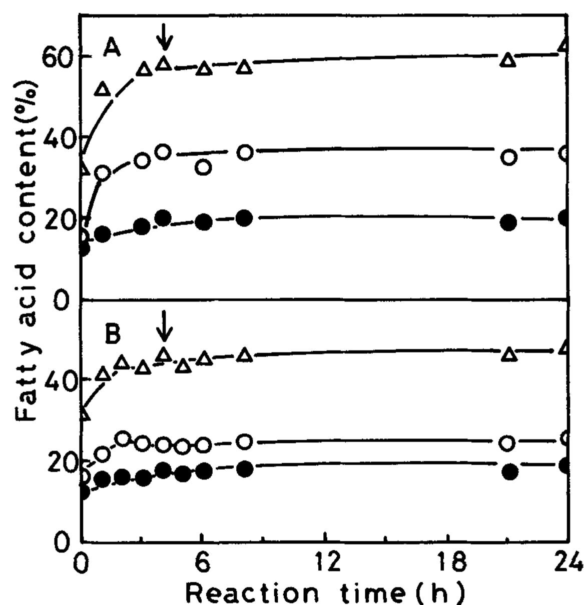

FIG. 4. Two-stage acidolysis of refined sardine oil with n-3 polyunsaturated fatty acid (PUFA) (time course of n-3 PUFA incorporation). Lyophilized immobilized enzyme was used. Refined sardine oil, n-3 PUFA; A, 1:4; B, 1:1. Arrows indicate the time when vacuum was started. ○, Eicosapentaenoic acid; ●, docosahexaenoic acid; △, n-3 PUFA.

TABLE 1

Moisture Content of the Reaction Mixture Used in Figure 2

Time	Moisture content ($\times 10^{-2}$%) Under CO_2 gas	In vacuum
Before the start of reaction	4.03	4.03
10 min after the start of reaction	9.28	4.63
48 h after the start of reaction	11.3	1.55

the necessary amount for enzymic catalysis. One of the authors reported previously (10) the importance of a trace amount of water for enzymic catalysis in organic solvent.

Two-stage acidolysis reaction of RSO under controlled water content. The results in Figures 2 and 3 suggested that the reaction under CO_2 at the early stage increased the reaction rate, and the succeeding reaction in vacuum to reduce DG formation would enhance the performance of the acidolysis reaction. Figure 4 shows the acidolysis of RSO carried out under CO_2 replacement during the early period (about 3 h) and then carried out in vacuum at 5–24 h. EPA incorporation in RSO was detected constantly, and the increase in DHA was small (Fig. 4). During the early period, both DG production and an increase in FFA content, which were due to hydrolysis, were detected. When the vacuum was started, DG content was reduced and TG was synthesized because of a decrease in the water content in the reaction mixture (Fig. 5).

Effects of initial water content of reaction mixture on acidolysis of RSO. From the results shown in Figures 2–5, it was thought that the initial water content of the reaction mixture was one of the important factors affecting acidolysis. The previously lyophilized immobilized enzyme was used for the data in Figures 4 and 5. Acidolysis was carried out with RSO containing various amounts of water and the lyophilized immobilized enzyme. The addition of water to the reaction mixture resulted in higher

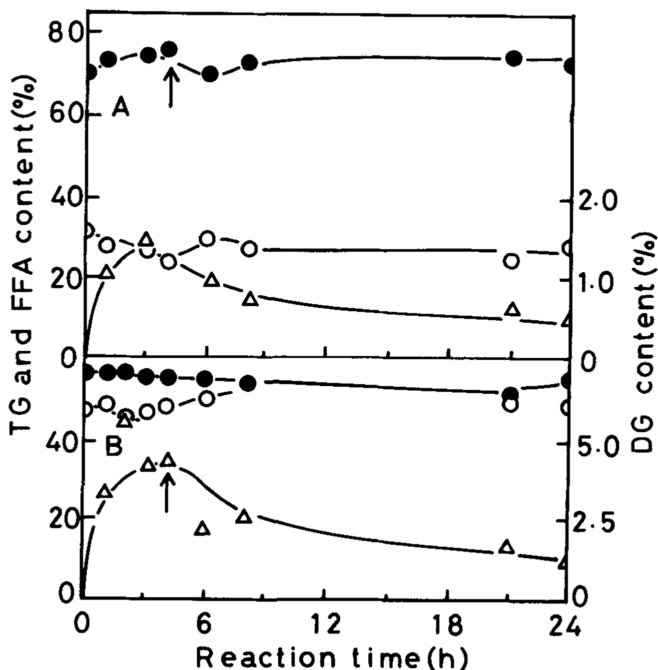

FIG. 5. Two-stage acidolysis of refined sardine oil with n-3 polyunsaturated fatty acid (PUFA) (time course of lipid components). Lyophilized immobilized enzyme was used. Refined sardine oil, n-3 PUFA; A, 1:4; B, 1:1. Arrows indicate the time when vacuum was started. ○, Triglyceride; ●, free fatty acid; △, diglyceride.

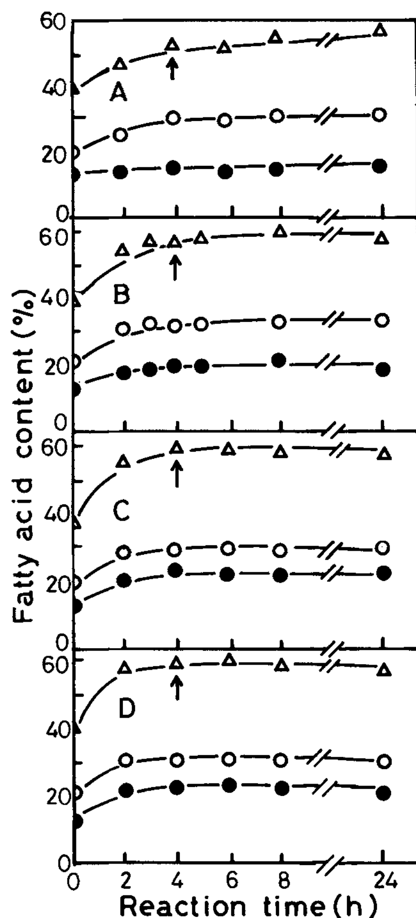

FIG. 6. Effect of initial water content of reaction mixture on n-3 polyunsaturated fatty acid (PUFA) incorporation during acidolysis of refined sardine oil. Lyophilized immobilized enzyme was used. Refined sardine oil, n-3 PUFA (1:1). A, 0.04%; B, 0.09%; C, 0.22%; and D, 7.90%. Arrows indicate the time when vacuum was started. ○, Eicosapentaenoic acid; ●, docosahexaenoic acid; △, n-3 PUFA.

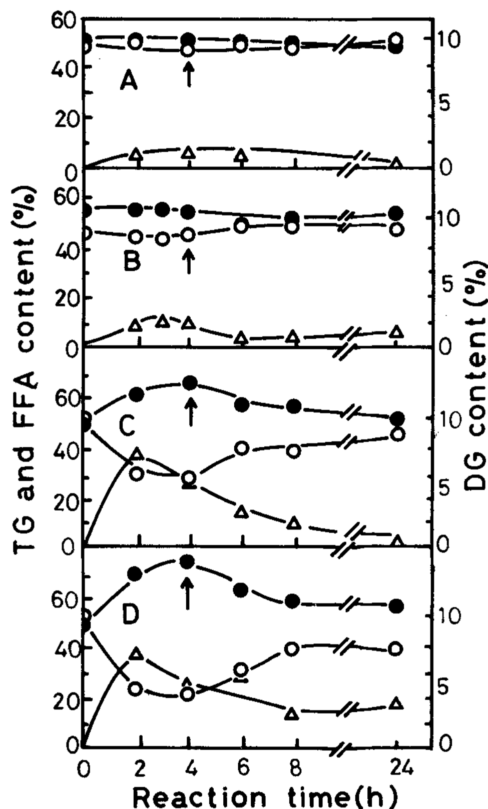

FIG. 7. Effect of initial water content of reaction mixture on the lipid components during acidolysis of refined sardine oil. Lyophilized immobilized enzyme was used. Refined sardine oil, n-3 polyunsaturated fatty acid (1:1). A, 0.04%; B, 0.09%; C, 0.22%; and D, 7.90%. Arrows indicate the time when vacuum was started. ○, Triglyceride; ●, free fatty acid; △, diglyceride.

reaction rates (Fig. 6). This result indicates that the addition of suitable amounts of water to the reaction mixture shortened the reaction period of the early stage. The recovery of TG was reduced when more water (7.9%) was added in the reaction mixture (Fig. 7), because too much DG was formed which was not completely converted to TG in the later stage.

Among the various methods to increase n-3 PUFA content of fish oil, acidolysis with free n-3 PUFA seems the most promising in terms of yield and subsequent separation of n-3 PUFA-enriched TG from the reaction mixture. However, performance of acidolysis (acidolysis rate, the final TG recovery, the final n-3 PUFA content, the final EPA and DHA contents, etc.) depends on various factors. The acidolysis rate generally increases with increasing free water content, as well as when the amount of immobilized enzyme is increased. Final TG recovery becomes higher when DG is converted to TG by esterification. The final content of n-3 PUFA increased with an increasing relative ratio of n-3 PUFA to fish oil, but the increase in the amount of n-3 PUFA is accompanied with a rise in cost. The two-stage acidolysis reaction, *i.e.*, first the reaction at appropriate moisture content followed by esterification in vacuum to convert DG to TG, seems the most promising.

ACKNOWLEDGMENTS

We are grateful to K. Osada and K. Maruyama, Taiyo Central R & D Institute, Taiyo Fisheries Co., Ltd., for the gift of free EPA.

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[Received March 13, 1992; accepted July 20, 1992]