SHORT COMMUNICATION

Increasing n-3 Polyunsaturated Fatty Acid Content of Fish Oil by Temperature Control of Lipase-Catalyzed Acidolysis[1]

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An attempt was made to further increase the content of n-3 polyunsaturated fatty acid (n-3 PUFA) of fish oil by lipase-catalyzed acidolysis (reaction between fish oil and n-3 PUFA-enriched free fatty acid) without solvent. A bioreactor system was constructed composed of a water-jacketed packed-bed column and a substrate reservoir with a circulation pipeline between the packed-bed column and the reservoir. By keeping the temperature of the reservoir at −10°C (for the first 20 h), followed by −20°C (for the subsequent 40 h) during the batch acidolysis, crystals of free fatty acid appeared, which were removed intermittently by a cotton plug packed in the tip of the outlet pipe in the reservoir. The n-3 PUFA content of the triacylglycerol fraction increased a further 10% by the reduced temperature of the reservoir.

KEY WORDS: Acidolysis, cod liver oil, docosahexaenoic acid, eicosapentaenoic acid, immobilized lipase, lipase, polyunsaturated fatty acids, PUFA-enrichment of fish oil, winterization.

n-3 Polyunsaturated fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have some pharmacological and physiological effects on human health (1,2). It is well known that EPA has an antithrombotic effect, and it has recently been demonstrated by animal tests that DHA has a positive effect on brain health.

Fish oils, such as cod liver oil and sardine oil, contain n-3 PUFA amounting to 20–30% of the total fatty acids (FAs). Among various fish oils, orbital fat of tuna or of fonito contains the highest amount of n-3 PUFA, up to 40% of the total FAs, but its production will be limited because of the low availability of orbital fat. By increasing the n-3 PUFA content of a fish oil, such as cod liver oil or sardine oil, one can take the required dose of n-3 PUFA (in the form of a gelatin capsule) without taking undesired FAs (mostly saturated FAs).

Several researchers, including us, have reported on the enrichment of n-3 PUFA in fish oil by means of acidolysis catalyzed by immobilized lipase (3–7). In our previous paper (5), we reported a preparation of fish oil rich in n-3 PUFA by nonsolvent lipase-catalyzed acidolysis. The reactions were simply carried out in a sealed flask containing both the reaction mixture and the immobilized lipase particles, which were agitated with a magnetic stirrer bar. Similar reactions were recently reported by Toyoshima *et al.* (6), indicating that the immobilized enzyme could be reused more than twenty times. During the batch reactions, the immobilized lipase particles were somewhat ruptured into smaller powders. In this article, we describe an industrially more feasible process composed of a packed-bed bioreactor, a reaction-mixture reservoir and substrate-circulation pipelines that connect the bioreactor and reservoir. We discovered that a further increase in n-3 PUFA content of triacylglycerol (TG) fraction is possible by chilling down the reservoir to a certain temperature.

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EXPERIMENTAL PROCEDURES

Fish oil and enzyme. Cod liver oil was obtained from Peter Moller a/s (Oslo, Norway). n-3 PUFA-enriched free fatty acid (FFA) was obtained from Central R & D Institute, Taiyo Fisheries Co., Ltd. (Tsukuba, Japan). FA compositions of the cod liver oil and FFA are shown in Table 1.

Immobilized *Mucor miehei* lipase (Lipozyme IM-60) was obtained from Novo Nordisk Bioindustry Co., Ltd. (Bagsvaard, Denmark). Its hydrolytic activity was 4.53 × 10^3 U/g, which was determined according to the method reported by Yamane (8).

Bioreactor and lipase-catalyzed acidolysis reaction. The bioreactor system constructed for this study is shown in Figure 1. Twelve grams of the immobilized lipase particles were packed in the water-jacketed column (1.5-cm i.d. and 17-cm length) that was kept at 40°C by circulating water. At the beginning of the reaction, the reaction mixture was prepared by mixing 60 mL cod liver oil, 60 mL FFA and 0.4 mL pure water and was poured into a reservoir made from a glass test tube (2.5-cm inside diameter and 15-cm length). Water was dispersed uniformly in the oil phase by ultrasonication. The reaction mixture was circulated from the reservoir into the bottom of the packed column and back from the top of the packed column to the reservoir with a peristaltic tubing pump (Model Minipuls 2; Gilson Co. Ltd., Villiers-le-Bel, France). The average flow rate was about 1 mL/min (actual value varied from 0.5 to 1.5 mL/min during the reaction because of changes in the

TABLE 1

Fatty Acid (FA) Compositions of Cod Liver Oil and n-3 PUFA-Enriched Free Fatty Acid (FFA)[a]

FA	Content (%)	
	Cod liver oil	n-3 PUFA-enriched FFA
14:0	4.0	1.3
16:0	11.5	0.4
16:1	8.5	1.3
18:0	1.7	2.4
18:1	22.8	2.5
18:2	1.9	1.9
20:1	14.1	13.1
22:1	9.6	1.3
20:5 (EPA)	8.7	34.5
22:5	1.2	1.7
22:6 (DHA)	13.1	36.0
Total n-3 PUFA	23.0	72.2

[a]Abbreviations: PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

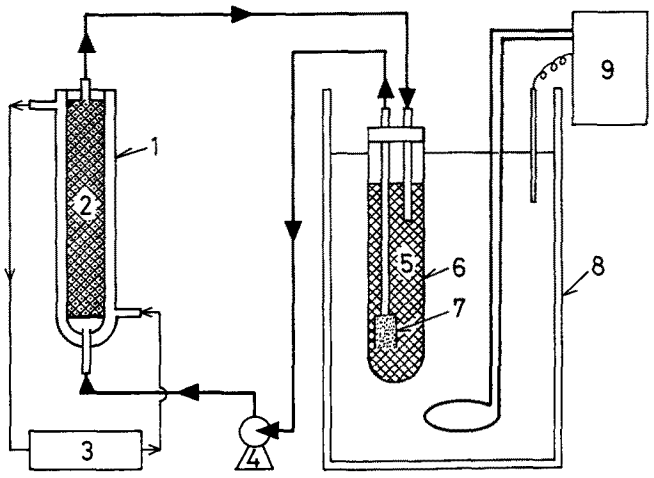

FIG. 1. Schematic diagram of the bioreactor system constructed for the reaction at lower temperature of the substrate reservoir. In the reactions with the substrate reservoir at room temperature, the substrate reservoir was not placed into the ethanolic water bath. 1, water-jacketed column; 2, immobilized lipase; 3, water bath (40°C); 4, peristaltic tubing pump; 5, the reaction mixture composed of cod liver oil and n-3 polyunsaturated fatty acid-enriched free fatty acid; 6, reservoir; 7, cotton; 8, ethanolic water (1:1 in vol) bath; 9, refrigerator.

elastic property of the Biton tubing and in the nature of the fluid). The reservoir was left at room temperature on a bench during the whole period of the reaction or was put into an ethanolic water bath (ethanol/water, 1:1, vol/vol), the temperature of which was controlled by a refrigerator unit. For experiments at chilled temperature, the tip of the outlet pipe in the reservoir was wider (2-cm width and 2-cm length) in which *ca.* 0.2 g cotton was packed to remove FAs crystallized during the reaction. The cotton plug must be replaced with new cotton to continue the circulation of the reaction mixture smoothly. The replacement was done every 5 h during the first 20 h for which the reservoir temperature was kept at −10°C, then every 8 h during the subsequent 30 h during which the reservoir temperature was kept at −20°C. After 50 h, the reaction mixture was further circulated for 10 h at −20°C without replacement of the cotton. The total reaction time was 60 h. Experiments were conducted in triplicate under the same reaction conditions.

Analytical procedures. Changes in lipid composition [TG, diacylglycerol (DG), monoacylglycerol and FFA] of the reaction mixture and in the FA composition of the TG fraction, as well as in the water content of the reaction mixture, were analyzed according to the methods described previously (5,9).

RESULTS AND DISCUSSION

A comparison of the time courses of EPA, DHA and total n-3 PUFA contents of the TG fraction for the reactions at room and chilled temperatures is shown in Figure 2. Differences in the content of n-3 PUFA at the two temperatures started to appear at 15 h and reached *ca.*

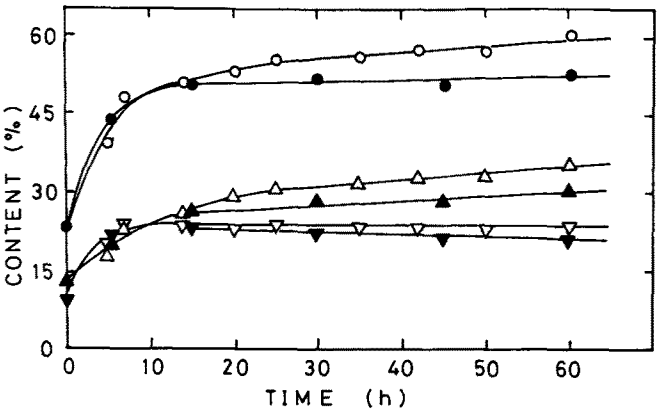

FIG. 2. Comparison of n-3 polyunsaturated fatty acids (PUFA) content of triacylglycerol fraction in terms of substrate circulation at room and chilled temperatures of the reservoir. Data at chilled temperature: ▽, eicosapentaenoic acid (EPA); △, docosahexaenoic acid (DHA); ○, total n-3 PUFA. Data at room temperature: ▼, EPA; ▲, DHA; ●, total n-3 PUFA.

TABLE 2

FA Compositions of TG and FFA Fractions of the Reaction Mixutre at the End of Batch Acidolysis[a]

	Content (%)					
	FFA			TG		
FA	Room temperature	Low temperature	Difference[b]	Room temperature	Low temperature	Difference[b]
14:0	3.6	2.6	−1.0	2.6	1.6	−1.0
16:0	4.6	0.6	−4.0	6.7	1.2	−5.5
16:1	6.4	5.8	−0.6	4.9	4.1	−0.8
18:0	0.5	0.8	0.3	0.9	0.6	−0.3
18:1	9.4	11.2	1.8	12.9	13.3	0.4
18:2	1.8	3.0	1.2	2.1	2.0	−0.1
20:1	4.6	3.9	−0.7	7.1	6.1	−1.0
22:1	2.7	2.2	−0.5	5.2	3.9	−1.3
20:5 (EPA)	25.3	26.1	0.8	19.0	22.4	3.4
22:5	1.3	1.3	0.0	1.7	1.9	0.2
22:6 (DHA)	28.1	29.1	1.0	28.3	34.3	6.0
Total PUFA	54.7	56.5	1.8	49.0	58.6	9.6

[a]Abbreviations: See Table 1; also TG, triacylglycerol.
[b]Difference = (content at low temperature) − (content at room temperature).

10% (*ca.* 3.4 and 6% in EPA and DHA contents, respectively). Continuation for more than 40 h showed no further increase in n-3 PUFA content, indicating that the reaction was in chemical equilibrium at 60 h. Stepwise or gradual chilling as described in Experimental Procedures was necessary because dropping the temperature of the reaction mixture down to −20°C from the beginning resulted in soft solidification of the reaction mixture, due to appearance of bulky crystals, which made its circulation impossible. Intermittent determination of DG content in the reaction mixture revealed that it increased steadily up to 2.5% at 60 h from 0% at the beginning. Almost the same results were obtained in the triplicate experiments. The contents of these DGs can be reduced by subsequent enzymatic reaction in vacuum (5).

The FA compositions of the FFA and TG fractions after 60 h for reactions at room and chilled temperatures are summarized in Table 2. The palmitic acid contents of FFA and of TG reduced from 4.6 to 0.6% and 6.7 to 1.2%, respectively. In addition to this, small reductions in the contents of $C_{14:0}$, $C_{16:1}$, $C_{20:1}$ and $C_{22:1}$ of FFA were observed. In contrast, the content of oleic acid ($C_{18:1}$), which was highest among the minor fatty acids of the FFA fraction, increased from 9.4 to 11.2%. Although the lowest temperature was −20°C in this work, because of the limited power of the refrigerator, it is expected that decreasing the temperature of the reservoir below −20°C will further increase the n-3 PUFA content if the problem of increased viscosity of the reaction mixture can be solved.

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