Enrichment of Polyunsaturated Fatty Acids from Sardine Cannery Effluents by Enzymatic Selective Esterification

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ABSTRACT: The sardine canning industry produces vast quantities of effluents that need expensive reprocessing. Their oily component contains valuable n-3 polyunsaturated fatty acids, namely EPA (5,8,11,14,17-eicosapentaenoic acid) and DHA (7,10,13,16,19-docosahexaenoic acid), up to 10% each. Our aim was to develop a process allowing the recovery of these fatty acids. After removing solid particles, proteins, and peptides from the crude effluent, the obtained oil was hydrolyzed. EPA and DHA were enriched from the recovered free fatty acid fraction by selective enzymatic esterification. Lipases were used as biocatalysts: Lipozyme™ allowed up to 80% DHA enrichment but gave no EPA enrichment. By immobilizing *Candida rugosa* lipase on Amberlite IRC50 cation-exchange resin, a 30% EPA enrichment was obtained.

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KEY WORDS: *Candida rugosa*, docosahexaenoic acid, eicosapentaenoic acid, fatty acid enrichment, fish oil, immobilized enzyme, lipase, polyunsaturated fatty acid, *Rhizomucor miehei*.

The n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are commonly known to play an important role in human health. Both are direct precursors of hormones regulating a wide range of biological functions such as blood pressure and inflammation (1–3). Therefore, they have a potential use for prevention and treatment of heart and circulatory diseases (4,5).

The main sources of EPA and DHA are fish oils, usually obtained as a by-product of fish meal processing. Sardine canning effluent can contain up to 70% oil. By extracting these valuable polyunsaturated fatty acids (PUFA) from this low-cost raw material, one can offset the cost of effluent reprocessing.

Several fractionation methods allow EPA and DHA recovery from fish oils. Physical and chemical methods use supercritical CO_2 (6,7), urea (8,9), low-temperature crystallization (10,11), or preparative high-performance liquid chromatography (HPLC) (11). More recently, enzymatic methods appeared (12–16), using lipases acting on DHA and EPA very weakly (17,18).

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Here we describe a continuous enrichment method for EPA and DHA in sardine oil using commercially available lipases as biocatalysts after optimization for batch reaction.

EXPERIMENTAL PROCEDURES

Materials. Sardine cannery effluent was from Les Fils de Dominique Ferrigno (Port Saint Louis-du-Rhône, France). *Candida rugosa* (lipase MY) was from Meito Sangyo Co. Ltd. (Aichi, Japan). Cation exchange resin Amberlite IRC50 was from Aldrich (Saint Quentin Fallavier, France). Lipozyme IM60 was a generous gift from Novo-Nordisk (Nanterre, France). Butanol was from Prolabo (Fontenay-sous-bois, France).

Preparation of free fatty acids from sardine oil. The effluent contained approximately 20% solid particles, 20% water, and 60% oil. After removing the solid particles (fish bones, flesh, and scales) by centrifugation (20 min, 6,000 × *g*, 25°C), and the water by decantation, the oil was washed twice with 5% phosphoric acid in order to remove phospholipids and dried with anhydrous sodium sulfate. Oil fractionation was achieved with a silica gel 60 column (Merck, Darmstadt, Germany). A fraction containing only triacylglycerol was obtained by elution with hexane/ether (87:13, vol/vol). The somewhat anomalous behavior of enzymes during sardine oil hydrolysis was discouraging. For this reason alkaline hydrolysis was carried out. The purified sardine oil was hydrolyzed at 64°C for 30 min in alcoholic KOH (1 N) under nitrogen. The resulting free fatty acids were kept at –18°C under nitrogen.

The fatty acid composition of sardine oil triacylglycerols was directly analyzed by gas–liquid chromatography (GLC) after methyl esterification. Peaks on GLC were able to identified by equivalent chain length value (ECL), with few unidentified minor peaks. The fish oil used contained saturated fatty acids (33.7%), monoenoic fatty acids (37.6%), dienoic fatty acids (2.7%), trienoic fatty acids (1.5%), and polyenoic fatty acids (with four, five, and six double bonds) (20.6%). Major fatty acids were palmitic acid (19.8%), EPA (11.9%), oleic acid (10.9%), DHA (9.5%), myristic acid (9.1%), and palmitoleic acid (8.2%). To eliminate most of those saturated fatty acids, a cold crystallization technique was employed, because the yield was better than for urea crystallization technique.

Saturated fatty acids elimination by low-temperature crystallization. Acetone (10 vol) was added to the fatty acid mix-

ture. The dissolved acetone solution was then cooled to –10°C, and kept for 8 h. The content of saturated fatty acids decreased to 13.8 from 33.7% owing to crystallization, and monounsaturated acids increased from 31.5 to 42.6%. The contents of dienoic and trienoic acids did not change. Polyenoic fatty acids (with four, five, and six double bonds) increased from 26.5 to 30.5%. The yield relative to the uncrystallized oil was 78% (vol/vol).

In that lot of oil after crystallization, the major fatty acids, in decreasing order were EPA (12.2%), DHA (11.7%), and oleic acid (11.6%).

Assay. Fatty acid analysis was performed using a Shimadzu CR 4A gas chromatograph (Kyoto, Japan) equipped with a split injector, a flame-ionization detector, and a Shimadzu integrator CR 3A. The methyl and butyl esters were separated on a BPX 70 column (0.22 mm × 25 m; SGE, Villeneuve Saint Georges, France). Nitrogen was used as carrier gas. The column temperature was raised from 150 to 220°C at 1.5°C/min. The injector and detector temperatures were 260 and 280°C, respectively. The methyl and butyl esters of fatty acids were identified by calculation of the ECL, using standard fatty acid esters (Sigma Chemical Co., Saint Quentin Fallavier, France).

Lipase activity was measured at 37°C and pH 7.0 under nitrogen flow by a Radiometer pH-stat (Copenhagen, Denmark), equipped with an automatic burette ABU 12, a titrator TTT2, a thermostated tank, and a Titrigraph recorder. The thermostated reaction vessel contained 40 mL buffer (35 mM Tris/HCl, 0.1 M NaCl, 5.0 mM $CaCl_2$, pH 7.0), 250 µL tributyrin from Fluka (Saint Quentin Fallavier, France), and 30 µL of the enzyme solution or 10 mg of immobilized enzyme preparation. Butyric acid liberated during hydrolysis was quantified by titration using a 35 mM sodium carbonate solution. One international unit (IU) of lipase was defined as the amount that liberated 1 µmol of butyric acid per minute.

Immobilization of C. rugosa *lipase.* Crude lipase powder (2.0 g) was dissolved in 20 mL of 50 mM sodium acetate buffer (pH 3.5) and centrifuged at 6,000 × *g* for 15 min at 4°C. The supernatant was desalted on a Sephadex G25 column (25 × 200 mm; Pharmacia Biotech; Orsay, France) using the same buffer. The active fraction was pooled (30 mL) and passed through 5.0 g of Amberlite IRC 50 resin that had previously soaked in the same buffer. Decrease in enzyme activity of the solution allowed monitoring of the immobilization process, which reached a plateau. The obtained enzyme-resin complex was rinsed with buffer and air-dried on a filter paper. The immobilization ratio was calculated as follows:

$$\text{immobilization (\%)} = [C \times D / (A - B)] \times 100 \qquad [1]$$

where A is the activity of the initial solution, B is the activity of the residual solution, C is the specific activity of the enzyme-resin complex, and D is the quantity of resin. Results about the immobilization are given in a previous publication (18).

Esterification. Reactions were conducted at 30°C in a rotatory shaker (250 rev/min) in 5-mL round-bottomed flasks. The reaction mixture was composed of 0.5 g (1.80 mmol) of fatty acids obtained by alkaline hydrolysis of fish oil and 0.135 g (1.80 mmol) of 1-butanol. When this mixture reached the desired temperature, 520 IU of crude *C. rugosa* lipase or immobilized lipase or Lipozyme was added, and the reaction mixture was incubated with shaking. Unesterified fatty acids were then esterified by diazomethane which was prepared according to the literature method (19). The methyl and butyl esters thus obtained were analyzed by GLC.

Esterification extent C was calculated as follows:

$$C = (A_b/\text{PM}_b)/(A_b/\text{PM}_b + A_x/\text{PM}_x) \qquad [2]$$

where A_b and A_x are the peak areas, and PM_b and PM_x are the molecular mass of the butyl and methyl esters, respectively.

Continuous esterification reaction. Continuous reaction was performed by feeding substrate to a fixed-bed reactor (1 g, 4,000 IU, 1.9 × 67 mm) with a peristaltic pump. The substrate mixture was composed of 15 g (0.055 mol) sardine free fatty acids and 8.2 g (0.110 mol) 1-butanol in 225 mL hexane.

RESULTS AND DISCUSSION

Esterification of fatty acids from sardine oil mediated by Lipozyme with 1-butanol. The fatty acids were esterified at 30°C for different times with equal or two molecular equivalents of 1-butanol in a mixture with or without *n*-hexane using Lipozyme as a catalyst. After 30 min, the esterification extents in all the reactions reached over 80% with slightly lower conversions in *n*-hexane (Fig. 1), owing to the effect of dilution of the mixture. After a certain point, the esterification's specificity decreased, and the higher the esterification extent, the lower the enrichment. So, notwithstanding reaction time and esterification extent, Table 1 shows the best enrichment of DHA and EPA in four kinds of reaction systems.

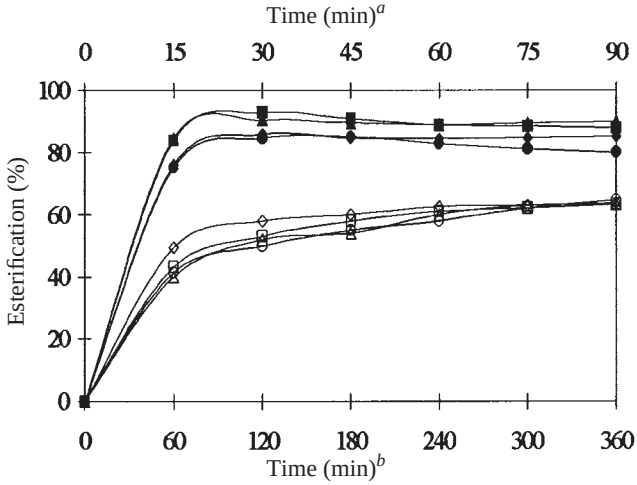

FIG. 1. Esterification of fatty acids prepared from sardine oil with 1-butanol. [a]Reaction using Lipozyme: ●, extent of esterification ratio 1:1 with *n*-hexane; ▲, ratio 1:1 without *n*-hexane; ◆, ratio 1:2 with *n*-hexane; ■, ratio 1:2 without *n*-hexane. [b]Reaction using *C. rugosa* lipase (Meito Sangyo Co. Ltd., Aichi, Japan): ○, ratio 1:1 with *n*-hexane; △, ratio 1:1 without *n*-hexane; ◇, ratio 1:2 with *n*-hexane; □, ratio 1:2 without *n*-hexane.

TABLE 1
Enrichment of Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) in Different Selective Esterification Systems

	Lipozyme[a]				*Candida rugosa* on IRC 50[a]			
	With *n*-hexane		Without *n*-hexane		With *n*-hexane		Without *n*-hexane	
	1:1[b]	1:2[c]	1:1[b]	1:2[c]	1:1[b]	1:2[c]	1:1[b]	1:2[c]
DHA enrichment[d]	3.7 ± 0.4	5.3 ± 0.5	5.5 ± 0.6	7.1 ± 0.7	3.4 ± 0.3	3.2 ± 0.3	3.8 ± 0.4	3.5 ± 0.3
DHA in the residual acid fraction (wt %)	43.3 ± 2.1	62.0 ± 3.1	64.3 ± 3.2	83.3 ± 4.2	39.8 ± 2.0	37.4 ± 2.0	44.5 ± 2.2	41.0 ± 2.0
EPA enrichment [e]	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	3.0 ± 0.3	2.6 ± 0.3	3.4 ± 0.3	2.8 ± 0.3
EPA in the residual acid fraction (wt %)	12.0 ± 0.6	11.9 ± 0.6	12.1 ± 0.6	12.0 ± 0.6	36.6 ± 1.8	31.7 ± 1.6	41.5 ± 2.0	34.2 ± 1.7

[a]Lipozyme (Novo Nordisk, Nanterre, France); *C. rugosa* lipase (Meito Sangyo Co. Ltd., Aichi, Japan); IRC 50 (Aldrich, Saint Quentin Fallavier, France).
[b]Free fatty acids/1-butanol (1:1, mol/mol).
[c]Free fatty acids/1-butanol (1:2, mol/mol).
[d]Percentage of DHA in the residual acid fraction divided by the percentage of DHA in the initial acid fraction.
[e]Percentage of EPA in the residual acid fraction divided by the percentage of EPA in the initial acid fraction.

When the fatty acids were esterified with an equal amount of 1-butanol in a mixture with *n*-hexane, DHA was obtained in a yield of 43.3% after 15 min, and the loss was 5.3% of the initial amount. In the esterification with an equimolar amount of 1-butanol without *n*-hexane, DHA was enriched to 64.3%. When the reaction was conducted with two molar equivalents of 1-butanol in a mixture without *n*-hexane, the DHA enrichment reached 7.1, which was equivalent to 83.3% of DHA in the unesterified fatty acid fraction. The loss of DHA was 3.5% of the initial amount. Lipozyme showed poor activity in esterifying DHA. However, the lipase acted on EPA somewhat, and the fatty acid could not enriched.

Selective esterification of sardine fatty acids with 1-butanol using C. rugosa *lipase powder.* When the selective esterification was carried out by a using commercial *C. rugosa* lipase powder, no fatty acid was esterified with 1-butanol. On the contrary, immobilization of this enzyme on resin enabled enzyme dispersion, and for this reason, enabled esterification.

Esterification mediated by C. rugosa *lipase immobilized on IRC 50 resin.* Four experiments were carried out for *C. rugosa* lipase immobilized on a cation exchange resin Amberlite IRC 50 in the same manners with Lipozyme as aforementioned. The activity of this immobilized enzyme was 5,000 IU/g. As illustrated in Figure 1, the ester yield was about 60%, regardless of the fatty acid/alcohol ratio. When the ratio was 1:2, the 60% yield level was achieved within 2 h, whereas it took 6 h when the ratio was equimolar (Fig. 1). Table 1 shows the best enrichment of DHA and EPA in four kinds of reaction system.

The best DHA enrichment was 3.8, corresponding to 44.5% of DHA in acid residual fraction, with a 1.1% loss. The DHA conversion rate was lower than 0.1. The EPA enrichment was 3.4, corresponding to 41.5% with a 2.8% loss. The conversion rate was higher than DHA (0.23). These results were obtained with an equimolar ratio of fatty acids/1-butanol in a reaction without *n*-hexane.

In reactions containing a ratio of 1:2 of fatty acids/1-butanol, the DHA enrichment was 3.5, corresponding to a rate of 41% of DHA in the acid residual fraction with a 0.7% loss and a conversion rate of 0.03. For EPA, the enrichment was 2.8, which corresponds to 34.2% in the fatty acid fraction with 3.9% loss. The conversion rate was 0.3.

Continuous selective esterification of fatty acids. Continuous selective esterification of the fatty acids was conducted with a fixed-bed reactor. The flow rate of the reaction mixture was maintained by a peristaltic pump. A mixture of fatty acids/1-butanol (1:2, mol/mol) was diluted with *n*-hexane in order to reduce the viscosity of the mixture. The substrate mixture was fed only one time.

When Lipozyme was used as the catalyst, the reaction was conducted using a fixed-bed reactor packed with Lipozyme (1 g, 1.9 × 67 mm). The effect of flow rate on the esterification extent is shown in Figure 2. The esterification extent increased as the flow rate decreased, and the extents at flow rates of 0.5 and 5 mL/h were 91 and 50% respectively. The extent of DHA and EPA unesterified fraction depended on the flow rate, and increased to 64.4 and 24.4% at flow rates of 0.5 mL/h, respectively.

We also carried out continuous flow reactions with immobilized *Candida* lipase. The reaction medium was the same as that of the reaction with Lipozyme. The quantity of butylic esters formed was low, as shown in Figure 3. The percentage

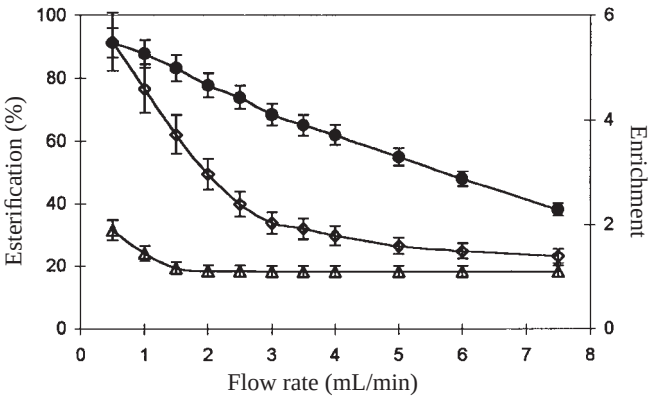

FIG. 2. Esterification of fatty acids prepared from sardine oil with 1-butanol in a continuous system using Lipozyme, and enrichment of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). ●, Extent of esterification; ◇, enrichment of DHA; △, enrichment of EPA.

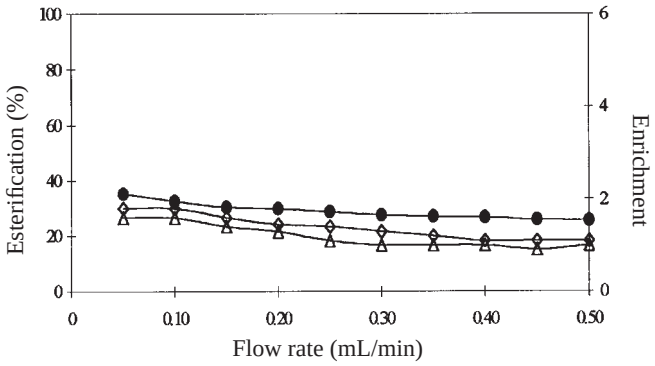

FIG. 3. Esterification of fatty acids prepared from sardine oil with 1-butanol in a continuous system using immobilized *C. rugosa* lipase and enrichment of DHA and EPA. ●, Extent of esterification; △, enrichment of DHA; ◇, enrichment of EPA. For abbreviations see Figures 1 and 2.

of esters did not exceed 38%. For this reason, DHA and EPA enrichment was not very substantial, owing to the low fatty acid esterification rate of the mixture.

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