MARINE BIOTECHNOLOGY

Proteolytic Extraction of Salmon Oil and PUFA Concentration by Lipases

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Received: 12 December 2003 / Accepted: 17 July 2004 / Online publication: 15 March 2005

Abstract

Commercial proteases (Alcalase®, Neutrase®, and Fla7vourzyme™) were tested for their ability to release the oil content of marine by-products (salmon heads). The amount of oil (17%) obtained after 2 hours was close to that obtained by the chemical extraction method (20%). Lipolysis of the oil was carried out with Novozym SP398 to obtain a mixture of free fatty acids and acylglycerols (24 hours 45% hydrolysis). The mixture was filtered on a hydrophobic membrane to discriminate between high melting saturated fatty acids and low melting acylglycerols. The sum of total polyunsaturated fatty acids increased from 41.6% in the crude oil to 46.5% in the permeate. The docosahexaenoic acid content increased from 9.9% to 11.6%, and the eicosapentaenoic acid changed from 3.6% to 5.6%. Data from differential scanning calorimetry DSC and from thin layer chromatography coupled with flame ionization detection (TLC-FID) differed significantly between permeate and retentate. A re-esterification of the free fatty acids in the permeate with Lipozyme IM was carried out to increase the amount of long chain acylglycerols.

Key words: fish by-products — hydrolysis — lipase — membrane fractionation — polyunsaturated fatty acid — differential scanning calorimetry

Introduction

The processes related to fish oil extraction a have been used for some time, as is evident in the years of several related patents (GB199054, Takahashi and Mitsui, 1923; GB438056, Fawcoti, 1935; GB590638, Fosse, 1947). They all rely on temperatures exceeding 95°C, resulting in protein coagulation and subsequent oil extraction by pressing the slurry. Because of the presence of long-chain polyunsaturated fatty acids (PUFAs) 20:5n-3 and 22:6n-3), degradation reactions such as oxidation are unavoidable. From a technological point of view, a number of studies have shown the incidence of PUFA in oxidative and hydrolytic alteration of food during processing (Neidleman, 1987; Ackman and Gunnlaugsdottir, 1991). However, more recent studies point out the importance of lowering the main parameters of temperature and time to reduce structural alterations (Chantachum et al., 2000; Aidos et al., 2003).

Enzymatic oil extraction using food-grade proteases could provide an interesting alternative. The use of these biotechnological tools for such a purpose takes place at a moderate temperature for a short duration (50°–60°C, 30–120 minutes). Furthermore, food-grade proteases allowing oil release from the protein matrix are not expensive and are widely used in the food industry. The resulting mixture can be easily separated by ultrafiltration, a process extensively used in the dairy industry.

Several commercial enzymes from animal, plant, or microbial sources can be used for this purpose, such as pepsine, papain, Alcalase, Neutrase, Corolase PN-L, Corolase 7089, Flavourzyme, and Protamex (Shahidi et al., 1995; Liaset et al, 2000, 2002; Kristinsson and Rasco, 2000b). The monitoring of proteolysis is of considerable importance for improving the physicochemical, functional, and sensory properties of the hydrolysates, without compromising the nutritive value of the proteic fraction (Kristinsson and Rasco, 2000a; Liaset et al., 2000). However, all these studies have focused on the properties of the protein hydrolysates, leaving the lipidic fraction aside. It is noteworthy that fish oils are an important source of PUFAs, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The realization that these fatty acids play an important role in hu-

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 DOI: 10.1007/s10126-004-0149-2 • Volume 15, 70–76 (2005) •

man health has led to much research into methods of extracting and concentrating these molecules from marine oils (Moore and McNeill, 1996). So far, few studies have been carried out on oil extraction processes under mild conditions (without the use of solvents). A combination of such mild conditions with solvent-free membrane filtration coupled with lipase treatments would compete with expensive current processes such as distillation, supercritical fluid extraction, crystallization in solvent, or urea complexation (Wanasundara and Shahidi, 1999; Xu, 2000). The technology is mature and already applied in the area of oils and fats, mainly in the degumming of vegetable oil (Bassam et al., 2001; Choukri et al, 2001; Xu et al., 2000).

As a first step, the aim of the present work was to test 3 food-grade proteases to recover the lipid fraction after centrifugation of the resulting slurry. To produce ''high added value'' lipid fractions, the oil was submitted to a lipase treatment (Novozym SP 398) followed by membrane filtration in order to discriminate between the mixture of free fatty acids and acylglycerols according to their melting points. Subsequently, the filtered oil (permeate) was re-esterified with an immobilized 1,3-specific lipase IM60 (Lipozyme IM) to enrich the newly formed acylglycerols in PUFA.

Materials and Methods

Materials and Reagents. Salmon by-products (100 kg of heads) obtained from a fish processing plant were ground and homogenized at 4°C using a Britek TR 130 grinder, immediately before analysis. All chemicals and reagents were food-grade. Alcalase 2.4L, Neutrase 0.5L, and Flavourzyme were obtained from Novo Enzymes. Lipid hydrolysis was carried out with a lipase from *Aspergillus oryzae* (Novozym SP 398), and an immobilized 1,3-specific lipase IM60 (Lipozyme IM) was used for re-esterification. Novo Enzymes also supplied both lipases.

Analysis. Water content was determined by oven-drying approximately 5 g of the sample at 105°C until constant weight was achieved (AOAC, 1999). Total lipids were extracted from the by-products according to the method of Bligh and Dyer (1959). The total crude protein content of samples was determined using the Kjeldahl method (AOAC, 1999). The ash content was determined by heating in a muffle furnace at 550°C (AOAC, 1999). Peroxide, iodine, and saponification values were quantified according to the AOAC 1999 procedures (965.33, 993.20, and 920.160, respectively). All assays were carried out in triplicate.

FAME Analysis by GLC. The fatty acid compositions of lipid fractions were investigated after conversion to their fatty acid methyl esters (FAMEs) using the boron trifluoride–methanol method (Ackman, 1998). An internal standard containing methyl tridecanoate, methyl pentadecanoate, methyl nonadecanoate, and methyl heneicosanoate was added (FAME Mix GLC-90, Supelco), and 1 μl of 2 mg/ml hexane FAME was injected into the gas chromatograph. Analytical GLC of FAME was carried out in a Perichrom 2000 system equipped with a flame ionization detector (FID) and a fused silica capillary column (30-m × 0.22-mm i.d. × 0.25-μm film thickness, BPX70 SGE Australia Pty. Ltd., analytical products). Nitrogen was used as the carrier gas. A temperature program set at 145°C for 5 minutes, rising to 210°C at a rate of 2°C/min. and held at 210°C for 10 minutes was used. The injector and detector temperatures were both set at 260°C. Fatty acids were identified by comparing their retention times with standard mixtures (PUFA 1 from marine source and PUFA 2 from animal source; Supelco. Peak areas were automatically integrated using the Perichrom chromatography data software, and individual fatty acids were reported as area percentage. All assays were carried out in duplicate.

Thermal Profiles of Oil Fractions. Thermal profiles of salmon oil fractions were generated with a differential scanning calorimeter (DSC; Pyris 1, PerkinElmer). Samples were cooled under nitrogen from room temperature to −65°C, which was held for 10 minutes to allow for polymorphic changes, and finally heated at 5°C/min up to 80°C. The heat-of-fusion enthalpies ΔH (J/g) of the thermograms were calculated for each peak from −65°C to +40°C using the Pyris software (PerkinElmer).

TLC-FID Analysis. The lipid classes of the different fractions were determined by thin-layer chromatography coupled with flame ionization detection (TLC-FID) using the Iatroscan MK-5 apparatus (Iatron Laboratories). Five independent analyses were performed for each fraction. An aliquot (1–3 μl) of lipids was spotted on quartz rods coated with silica gel (Chromarods S-III, Iatron Laboratories). The rods were developed in hexane, diethyl ether, and formic acid (98:2:1 v/v/v) for 40 minutes, dried in an oven at 110°C, then scanned in the Iatroscan analyzer. The Iatroscan was operated under the following conditions: flow rate of hydrogen, 160 ml/min; flow rate of air, 2 L/min. The ChromStar internal software provided the recording and integration of the peaks.

Enzymatic Hydrolysis. Three commercial proteases were tested for their ability to release the oil content of by-products. Protein hydrolysis was carried out in a thermostated batch reactor equipped with an overhead stirrer, under nitrogen flux. Three batches containing 10 kg of crushed by-products were subjected to the action of Neutrase 0.5L at 45°C, pH 7.0, Alcalase 2.4L at 55°C, pH 7.5, and Flavourzyme at 50°C, pH 7.5, for 2 hours. Each batch of 10 kg contained 18% protein (N × 6.25). The ratio of enzyme to substrate (E/S) was set at 0.05 for each enzyme assayed, that is, 90 g of enzyme per 10 kg of salmon by-products. Reaction kinetics were monitored by measuring the degree of hydrolysis using the pH-stat method according to the procedure of Adler-Nissen (1986). The equation relating DH to the alkali consumption during the course of the hydrolysis reaction is given by

$$DH(\%) = \frac{1}{\alpha \cdot h_{\text{tot}}} \cdot \frac{B \cdot N_{\text{B}}}{MP} \cdot 100,$$

where DH is defined as the rate of the number of peptide bonds cleaved over the total number of such bonds in the protein substrate (h_{tot}), which is calculated from the amino acid composition of the substrate and can be approximated to 7.5 equivalent per kilogram of protein (calculated as Kjeldahl N × 6.25); B is the alkali consumption in milliliters; N_{B} is the normality of the alkali (2 N NaOH); MP refers to the grams of protein (N × 6.25) in the reactor.

After this, the reaction mixture was cooled at 4°C using a pilot frame exchanger before centrifugation with a Westfalia Separator. Samples (400 g) of the reaction mixture were withdrawn for analysis and centrifuged to recover the oil (5000 × g under nitrogen for 10 minutes at 4°C in a centrifuge (J2-HS, Beckman Instruments). The protease remaining in the aqueous fraction was inactivated by live steam injection.The degree of dissociation is

$$\alpha = \frac{10^{\text{pH}-\text{pK}}}{1 + 10^{\text{pH}-\text{pK}}}$$

The pK values at different temperatures T (Kelvin) were calculated from Steinhardt and Beychok (1964):

$$\text{pK} = 7.8 + \frac{(2.98 - T)}{298 \cdot T} \cdot 2400$$

Oil Hydrolysis with Lipase. Lipase hydrolysis was earned out in a 2000-ml jacketed glass reactor blanketed with nitrogen and kept sealed throughout the reaction at 37°C, pH 7.0, for 12 hours under stirring at 800 rpm. A reaction mixture containing 1000 g of oil, 200 kilo lipase units (KLU), and 1000 ml of distilled water was incubated. The hydrolysis percentage of the oil, after enzymatic treatment, was measured according to the following equation: where the acid value is expressed as milligrams of KOH required to neutralize free fatty acids present in 1 g of oil; the saponification value is defined as

$$\begin{aligned}\text{Hydrolysis}\,(\%) &= [(\text{Acid Value}_{(\text{hydrolyzed oil})} \\ &\quad - \text{Acid Value}_{(\text{unhydrolyzed oil})}) \\ &\quad \div (\text{Saponification Value}_{(\text{unhydrolyzed oil})} \\ &\quad - \text{Acid Value}_{(\text{unhydrolyzed oil})})]\end{aligned}$$

milligrams of KOH required to saponify 1 g of oil. After centrifugation, the aqueous phase was removed from the hydrolyzed oil.

Membrane Filtration. The mixture of acylglycerol (AG) and free fatty acids (FFA) was subjected to filtration using a 150-mm length × 15-mm i.d. tubular ceramic membrane device with a porosity of 50 nm (Membralox, polyphosphazene ceramic tubular membrane, 1 bar, 24 hours, + 4°C). The driving pressure was set at 1 bar, and the mixture was circulated using a magnet drive gear pump, MG 200. The flow sheet to obtain a PUFA concentrate is shown in Figure 1.

Re-esterification Step. Permeate samples (5 g) were re-esterified with 5 g of glycerol using immobilized lipase (0.5 g of Lipozyme IM 60) and 100 μl distilled water at 50°C for 48 hours. Molecular sieves were added after 3 hours of incubation to remove the water formed during the reaction. The flasks were blanketed with nitrogen and kept sealed throughout the reaction. The vessels were swirled on an orbital shaker at 300 rpm. The degree of esterification was calculated from the amount of fatty acids consumed during the reaction, according to the procedure of He and Shahidi (1997). All experiments were carried out in triplicate. The enzymes were inactivated by live steam injection to recover fish hydrolysate (Figure 1).

Results and Discussion

Crude salmon heads contained (%): 38.2 ± 1.3 dry matter; 12.0 ± 0.3 protein; 20.0 ± 0.4 total lipid, and 2.6 ± 0.6 ash. The oil extracted showed 0.94% ± 0.04% oleic acidity; 240 ± 1 saponification value; 135.5 ± 2.6 iodine value; 2.4 ± 0.2 meq/kg peroxide value.

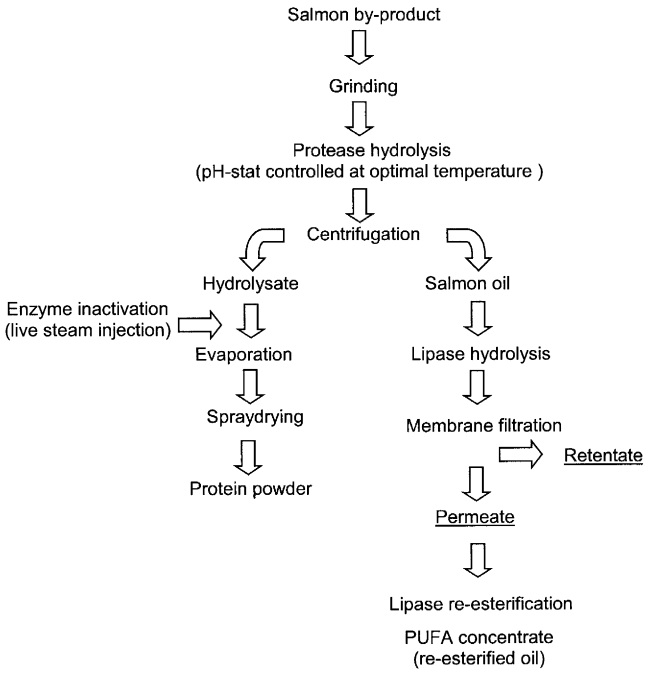

Fig. 1. Flow sheet for the production of salmon protein hydrolysate and crude oil.

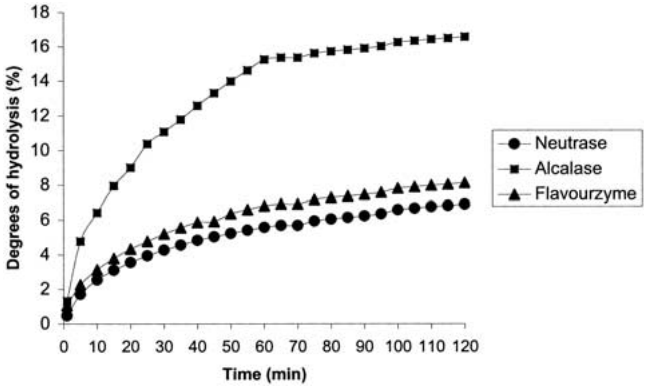

Fig. 2. Enzymatic hydrolysis of salmon by-products (heads) by Neutrase 0.5L (45°C, pH 7.0, E/S 0.5), Alcalase 2.4L (55°C, pH 7.5, E/S 0.5), and Flavourzyme (50°C, pH 7.5, E/S 0.5) for 2 hours.

The increase in *DH* (Figure 2) initially occurs rapidly as loosely bound polypeptide chains are cleaved from an insoluble protein particle. A second, slower reaction period occurs in which the more compact core protein is digested. After 2 hours of proteolysis with Neutrase, Flavourzyme, or Alcalase, the *DH* was in the range of 6.9–7.2, 8.0–8.2, or 16.4–16.6, respectively. The fat recoveries did not increase further with an increase in hydrolyzing time.

The final choice of Alcalase was based on cost considerations and on its specificity for terminal hydrophobic amino acids, which generally leads to the production of nonbitter hydrolysate (Adler-Nissen, 1986).

The fat was decanted from the aqueous phase after centrifugation, and no other removal was necessary with Alcalase, unlike the other 2 proteases. However, no significant difference was found between the 3 enzymes (Neutrase, 17.2%; Flavourzyme, 17.0%; Alcalase, 17.4%), whereas the chemical extraction (Bligh and Dyer, 1959) led to a better recovery (20%). Unlike current industrial processes (heating over 90°C for 30–90 minutes), our oil extraction was carried out under mild temperature conditions (below 55°C). After the centrifugation step, the enzymes were inactivated in the hydrolysates by live steam injection. The production of the $\omega 3$ concentrate from the salmon by-product by proteolysis was attempted via lipase action under mild conditions.

Hydrolysis with the lipase from *Aspergillus oryzae* (Novozym SP 398) led to a mixture of free fatty acids and acylglycerols. The reaction was stopped when the degree of hydrolysis had reached 45% in 24 hours. After that, the kinetics approached a stationary phase in which no apparent hydrolysis took place. Then the hydrolyzed oil was filtered through hydrophobic membrane in order to discriminate between high melting saturated fatty acids remaining in the retentate and low melting PUFAs in the permeate. The general tendency of PUFA is to be esterified at the *sn-2* position of fish oil (Aursand et al., 1995; Nwosu and Boyd, 1997; Wanasundara and Shahidi, 1997), whereas these fatty acids are mainly located at the *sn-1* and *sn-3* positions in marine mammal lipids. The difference in fatty acid composition between the crude oil and the permeate, showed a decrease of the saturated fatty acid from 27.2% to 20.2%, mainly represented by 16:0 and 14:0 (Table 1). The sum of total PUFA increased from 41.6% in the crude oil to 46.5% in the permeate, with an increase of DHA from 9.9% to 11.6% and EPA from 3.6% to 5.6%. In the same way, DSC analysis of the different fractions showed differences in melting temperatures (Figure 3). The crude oil displayed 2 main peaks at −45.4°C and −8.2°C. The permeate resulting from membrane filtration of the acylglycerol mixture presented several new peaks at −52.1°C, −28.4°C, −21.8°C, and +13.8°C. The high melting point of the retentate (+36.8°C) is noted worthy.

Subsequent esterification of the permeate was achieved using glycerol and an immobilized stereospecific *sn-1, sn-3* lipase (Lipozyme IM) at 50°C for 48 hours. The system could be re-esterified to greater than 90% acylglycerols, leaving a system of triacylglycerols, 2-monoacylglycerols, and diacylglycerols (He and Shahidi, 1997; Xu et al., 2000).

Table 1. Main Fatty Acids (% of the total fatty acids) of Crude Oil and Permeate Obtained After Filtration and Lipase Action Using SP 398.

Fatty acid	*Crude oil*	*Permeate*	*Permeate re-esterified*
14:0	7.31 ± 0.09	6.21 ± 0.07	5.72 ± 0.05
16:0	15.72 ± 0.04	11.42 ± 0.03	10.70 ± 0.03
18:0	2.80 ± 0.03	2.32 ± 0.02	2.21 ± 0.02
Σ SFA	27.2	20.2	19.9
16:1ω7	7.91 ± 0.13	7.73 ± 0.15	7.65 ± 0.09
18:1ω9	16.45 ± 0.03	18.53 ± 0.03	18.61 ± 0.04
20:1ω9	4.62 ± 0.05	6.05 ± 0.05	6.20 ± 0.02
Σ MUFA	30.2	33.3	33.5
18:2ω6	2.20 ± 0.01	2.31 ± 0.01	2.33 ± 0.02
20:4ω6	1.72 ± 0.06	1.70 ± 0.04	1.72 ± 0.03
Σω6	5.7	5.8	5.7
18:3ω3	1.01 ± 0.05	0.94 ± 0.05	0.92 ± 0.05
18:4ω3	2.32 ± 0.05	2.15 ± 0.07	2.12 ± 0.05
20:4ω3	9.85 ± 0.04	10.61 ± 0.03	10.70 ± 0.04
20:5ω3	3.60 ± 0.05	5.64 ± 0.04	5.06 ± 0.04
22:5ω3	4.01 ± 0.05	4.36 ± 0.05	4.40 ± 0.05
22:6ω3	9.90 ± 0.04	11.63 ± 0.05	11.90 ± 0.04
Σω3	31.7	36.0	37.1
Others	4.2	4.7	3.7
ΣPUFA	41.6	46.5	46.5

[a]Data are given as mean ± sd (n = 3)

The different lipid classes were quantified using the Iatroscan TLC-FID technique (Figure 4). The crude oil displayed a single peak representing 99% of triacylglycerols. Following the first hydrolysis step by lipase Novozym SP 398, 35.2% of (FFAs) were liberated from acylglycerols. After the membrane filtration step, 45% of these FFAs were retained in the retentate. In the filtrate were found the low melting FFAs, mainly PUFAs, which were re-esterified by Lipozyme IM 60. Owing to thermodynamic limitations, the kinetics of the reaction did not allow total re-esterification. Consequently, 12% of fatty acids remained free in the medium after 48 hours. Because the major objective of the present work was to maximize PUFA content in the form of acylglycerols, the glycerol and the FFA were easily eliminated by washing and neutralization by sodium hydroxide.

A large fraction of the 45% of FFAs obtained by lipolysis was retained in the retentate. As observed in Figure 4, a mixture of acylglycerols (22% TAG, 20% DAG, and 40% MAG), and 12% FFA was obtained using this lipase, according to Esteban Cerdan et al. (1998). The final product would comply with physicochemical properties (surfactants) and nutritional specifications, since ω3 fatty acids present in fish oil are of major importance in the prevention or treatment of a range of human diseases or disorders (Simopoulos, 1991; Ackman, 1999; Horrocks and Yeo, 1999).

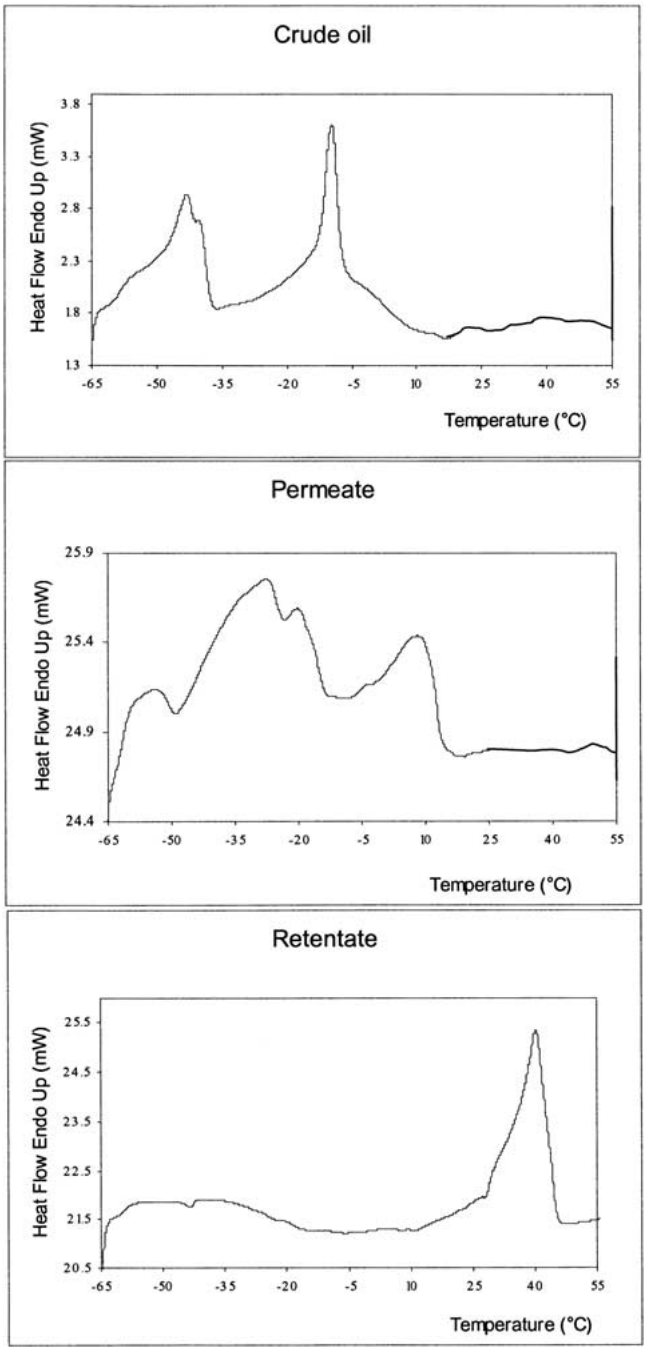

Fig. 3. Thermal behavior of salmon oil fractions obtained by differential scanning calorimetry. Operating conditions, –65°C to 80°C at 5°C/min; sample weight, 10 mg.

Conclusion

The present study tested a new method for oil extraction using biotechnological tools. This process has several advantages. It requires no organic solvent, low temperature, and use of commercial, low-cost, food-grade proteases, and oil is released in less than 2 hours. The method competes with current high-temperature, lengthy techniques, which use heating

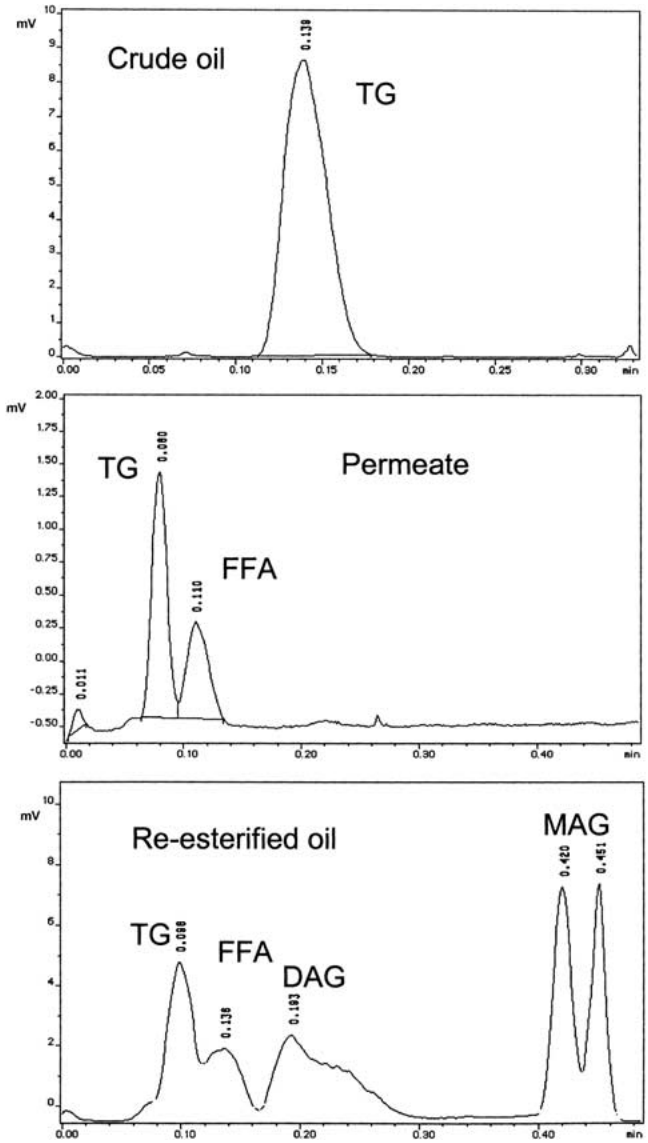

Fig. 4. Iatroscan TLC-FID chromatograms of salmon oil, permeate, and re-esterified permeate. Operating conditions: Chromarods SIII, 1μl development in hexane, diethyl ether, and formic acid (98:2:1, v/v/v) for 40 minutes. TG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; FFA, free fatty acid.

and pressure steps that have remained the same for 70 years. However, it should be stressed that this process can only be efficient with oily proteinic substrates, such as fish and meat by-products. In the case of plants, the presence of the cell walls, cellulose, and lignin prevent optimal oil release.

New processes excluding organic solvents are developing in the food, cosmetic, and nutraceutic areas. From this point of view, the use of lipases assisted by membrane filtration is an alternative method to increase the PUFA content of marine lipids for possible specific applications.

Acknowledgments

The authors thank ''Les Salaisons Maritimes André Ledun'' (Cany-Barville, France) for financial support.

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