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Enrichment of Eicosapentaenoic Acid from Sardine Oil with Δ5-Olefinic Bond Specific Lipase from *Bacillus licheniformis* MTCC 6824

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Lipase derived from *Bacillus licheniformis* MTCC 6824 was purified to homogeneity by anion exchange chromatography on Amberlite IRA 410 (Cl^-) and gel filtration using Sephadex G-100 as judged by denaturing polyacrylamide gel electrophoresis. The purified lipase was used for hydrolysis of triacylglycerol in sardine oil to enrich Δ5-polyunsaturated fatty acids (Δ5-PUFAs) namely, arachidonic acid (5,8,11,14-eicosatetraenoic acid, ARA, 20:4n-6) and eicosapentaenoic acid (5,8,11,14,17-eicosapentaenoic acid, EPA, 20:5n-3). The individual fatty acids were determined as fatty acid methyl esters (FAMEs) by gas–liquid chromatography and gas chromatography–mass spectroscopy as FAMEs and *N*-acyl pyrrolidides. The enzyme exhibited hydrolytic resistance toward ester bonds of Δ5-PUFAs as compared to those of other fatty acids and was proved to be effective for increasing the concentration of EPA and ARA from sardine oil. Utilizing this fatty acid specificity, EPA and ARA from sardine oil were enriched by lipase-mediated hydrolysis followed by urea fractionation at 4 °C. The purified lipase produced the highest degree of hydrolysis for SFAs and MUFAs (81.5 and 72.3%, respectively, from their initial content in sardine oil) after 9 h. The profile of conversion by lipase catalysis showed a steady increase up to 6 h and thereafter plateaued down. Lipase-catalyzed hydrolysis of sardine oil followed by urea adduction with methanol provided free fatty acids containing 55.4% EPA and 5.8% ARA, respectively, after complexation of saturated and less unsaturated fatty acids. The combination of enzymatic hydrolysis and urea complexation proved to be a promising method to obtain highly concentrated EPA and ARA from sardine oil.

KEYWORDS: Δ5-Olefinic bond specific lipase; sardine oil; PUFAs; EPA; ARA

INTRODUCTION

The n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFAs), namely, eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3), and arachidonic acid (ARA, 20:4n-6), are essential fatty acids in the diet of a majority of marine finfish and crustaceans, especially for the larvae and broodstock, because of their inability to synthesize de novo these fatty acids in adequate levels from precursor molecules (*1, 2*). The essential LC-PUFAs are also recognized to have beneficial therapeutic, physiological, and nutritional effects on human health (*3–7*). Most of the technologies available for purifying individual PUFAs and PUFA concentrates from fish oil are based on differences in physicochemical properties associated with the number of double bonds in the molecule or acyl chain length such as the urea complexation method, which is based on free fatty acids (*8*). Lipases [triacylglycerol acyl hydrolases (EC 3.1.1.3)] are one of the most important classes of hydrolytic enzymes that selectively catalyze the hydrolysis of triacylglycerols into free fatty acids, partial acylglycerols, and glycerol (*9–11*). Lipases occur widely in animals, plants, and microorganisms (*12, 13*). Among microbial lipases, bacterial lipases are the most widely used class of enzymes in biotechnological applications because of their higher stability compared with animal or plant lipases. The *Bacillus* lipases constitute a major group, and they have been reported from *B. coagulans* NCIMB 9365 (*14*) and *B. circulans* (*15*), which possess intracellular lipases, whereas a recently reported thermophilic *B. coagulans* BTS-3 isolate possessed an extracellular alkalophilic lipase (*16*). A typical example of lipase-mediated modification of fats and oils is enrichment of PUFAs (*17*). Lipases generally discriminate between PUFAs and other fatty acids and show less hydrolytic activity toward ester bonds of PUFAs. This makes the enzymes useful for concentrating PUFAs. Chemical hydrolysis may partially destroy the natural *all-cis* PUFA if the process is inadequately effected, due to the high temperatures involved (*18*). The mild conditions used in enzymatic reactions offer a promising alternative to chemical hydrolysis to avoid the oxidation and cis–trans isomerization. Earlier studies indicated that the rate of lipase catalysis is quite sensitive to the double-bond position within fatty acid groups (*17*). In most cases, the

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rate is lower when the double bonds are located near the carboxyl terminus. This type of substrate specificity has been utilized for the recovery of EPA (Δ5) and DHA (Δ4) from marine oils and γ-linolenic acid (Δ6) from borage seed oil (*19, 20*). There are also reports of lipases from *Chromobacterium viscosum* and *Pseudomonas* sp. releasing both fatty acids (*21–23*). As fish oils contain mixtures of EPA, DHA, and other unsaturated and saturated fatty acids (*24*), there is an urgent need to obtain purified PUFA concentrates, preferably after enzymatic hydrolysis of the oils. Most of the existing chemical purification methods are based on the hydrolysis of oils to free fatty acids, which are nonselective to different fatty acids and bring all fatty acids in solution form. The unique characteristics of lipases, that is, positional (acyl side chain and olefinic double bonds) and stereospecificity, can be utilized to selectively concentrate targeted fatty acids in triacylglycerol that can be readily absorbed into plasma triacylglycerol (*3, 25, 26*).

In this paper we report a method for the enrichment of Δ5 fatty acids, namely, EPA and ARA, from sardine oil in a one-step hydrolysis by *Bacillus licheniformis* MTCC 6824 alkaline lipase and further concentration of the fatty acids by urea complexation.

MATERIALS AND METHODS

Chemicals and Reagents. The solvents used for sample preparation were of analytical grade (E-Merck, Darmstadt, Germany) and were redistilled in an all-glass system. Double-distilled water was used throughout this work. Other chemical reagents, namely, methanol, *n*-hexane, NaOH, Na_2EDTA, HCl, urea, and molecular sieve, were obtained from Qualigens (India). Amberlite IRA 410 (Cl^- form) and bovine serum albumin were obtained from HiMedia (Mumbai, India); Sephadex G-100 and other supports of chromatography were from Sisco Research Laboratories (SRL, Mumbai, India). Electrophoresis grade acrylamide, bis-acrylamide, medium-range molecular marker proteins, and Coomassie Brilliant Blue R-250 were procured from Bangalore Genei (Bangalore, India). All glassware was rinsed with $CHCl_3/CH_3OH$ (2: l, v/v) and dried under N_2. Standards of fatty acid methyl ester (Supelco TM 37 Component FAME Mix) and boron trifluoride/methanol (14% BF_3 in methanol, w/v) were procured from Sigma-Aldrich Chemical Co. Inc. (St. Louis, MO). Fresh crude oil from whole sardine (*Sardinella longiceps*) was obtained from a plant located in Cochin, India, bleached with 4% activated charcoal, and stabilized with butylated hydroxyquinone (TBHQ, 0.01% w/v). The oil was stored under nitrogen at −20 °C, in a sealed dark amber glass container, until use. All reactions were monitored by UV fluorescence or staining with iodine or 2,7-dichlorofluorescein (0.1%) in methanol. GLC data were recorded on a Perkin-Elmer AutoSystem XL gas chromatograph. Mass spectral assays were obtained using a Varian 1200L single-quadruple mass spectrometer instrument under electron impact (EI, ionization energy = 70 eV) condition.

Purification of Lipase from *B. licheniformis* MTCC 6824. *B. licheniformis*MTCC 6824 was inoculated in an Erlenmeyer flask containing nutrient broth supplemented with $CaCl_2$ (0.05% w/v) and sardine oil (1.0% v/v, emulsified with Tween 80) as lipidic carbon sources (*13*). The culture broth obtained after incubation of the content (at 37 °C for 48 h) was centrifuged (10000 rpm for 20 min at 4 °C) to recover the supernatant, which was analyzed for lipase activity. The cell-free supernatant (500 mL) was concentrated to 50 mL by lyophilization (model Alpha 1-4LD, Martin Christ), followed by dialysis against Tris-HCl buffer (10 mM, pH 8.0). This concentrated liquid referred to as the crude lipase solution was used for further purification.

Briefly, a precipitate was obtained after the addition of $(NH_4)_2SO_4$ (70% saturation) to the crude enzyme solution at 4 °C. The precipitate after separation by centrifugation (15000 rpm, 30 min, 4 °C) was dissolved in Tris-HCl (50 mL, 50 mM, pH 8.0), to which ethanol/ether (4 °C, 100 mL, 1:1 v/v) was slowly added to obtain a precipitate. The excess solvent was evaporated by drying in vacuo at 0 °C. The residue was dialyzed against Tris-HCl (100 mL, 50 mM, pH 8.0), and the dialyzed material was centrifuged (10000*g*, 30 min, 4 °C) to obtain a supernatant (40 mL) to be used for anion exchange chromatographic purification on an Amberlite IRA 410 (Cl^- form) column equilibrated with Tris-HCl buffer (10 mM, pH 8.0). The enzyme was eluted with 0–0.5 M NaCI/10 mM Tris-HCl (pH 8.0) in a linear gradient. The fractions showing lipase activity (10 mL) were pooled and concentrated by lyophilization. The pooled and concentrated active fractions (3 mL) obtained after the previous step were rechromatogaphed on a Sephadex G-100 gel exclusion column equilibrated with Tris-HCl buffer (20 mM, pH 8.0) containing $CaCl_2$ (1.0 mM). The eluants from the chromatographic column were analyzed for total protein (A_{280}) and lipase activity (*13*). The lipolytic activity of the purified enzyme was estimated by a spectrophotometric method in a UV–VIS spectrophotometer (Varian Cary 50 Conc.) using 4-nitrophenyl palmitate (4-NPP) as substrate following established procedures (*27*). One activity unit of lipase (LU) was defined as micromolar 4-NP released from the hydrolysis of 4-NPP per milliliter per minute by 1 mL of enzyme at 45 °C under standard assay conditions. The protein concentration was determined by measuring the absorbance at 590 nm (Varian Cary 50 Conc.) according to Bradford's dye binding assay method using bovine serum albumin (20–150 μg) as a standard (*28*).

Polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (0.1% w/v SDS-PAGE) was carried out to establish the purity of the lipase protein following an established procedure (*29*). The SDS-PAGE was performed on 12% polyacrylamide gel (with 6% stacking gel), and the relative molecular mass of proteins was determined with reference to the medium-range molecular mass markers (14.4–94.0 kDa, Bangalore Genei, India). The molecular mass markers used were phosphorylase β (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa). After migration, the gels were fixed using 7% (v/v) CH_3COOH/CH_3OH and submitted to a cycle of staining/destaining with Coomassie Brilliant Blue R-250 and 14% (v/v) CH_3COOH/CH_3OH, respectively, to allow determination of the molecular mass of proteins (*13*). Molecular mass was determined from the plots of log molecular mass (log *M*) versus migration (R_f) for a series of known protein standards.

Preparation of Δ5-PUFA Concentrates by Lipase-Catalyzed Hydrolysis. Sardine oil (150 mL, added with 0.01% w/w *tert*-butylhydroquinone, TBHQ) and PIPES/NaOH buffer (150 mL of a 0.1 M solution, pH 7.0, and 0.7% v/v Triton X-100) supplemented with $CaCl_2$ (0.33 mL, 100 mM) were placed in a 50 mL screw-cap round-bottom flask containing glass beads following the procedure adopted by Tanaka with modification (*22*). Purified lipase (300 U) was added to the reaction mixture to initiate the hydrolysis. The round-bottom flasks were flushed with N_2 to prevent oxidation and placed into a shaker incubator at 45 ± 1 °C with magnetic stirring (500 rpm). Samples (0.5 mL) from the reaction mixture were withdrawn periodically for determining the lipase activity. Methanolic KOH (0.5 N, 25 mL) solution was added to the mixture to neutralize the free fatty acids released during hydrolysis. Distilled water (480 mL) was added to the reaction mixture, the lower aqueous layer was acidified to pH 1.0 with 2 N HCl (20 mL), and the triacylglycerols were extracted with *n*-hexane (100 mL × 2). The lower aqueous layer was discarded, and the upper *n*-hexane layer containing triacylglycerol was further extracted with distilled water (50 mL × 3) to remove free fatty acids. The upper *n*-hexane layer was concentrated at 40 °C, using a rotary evaporator. The concentrated glycerides were maintained under N_2 at −20 °C until further use.

The hydrolysis products of the reaction catalyzed by lipase were monitored by thin layer chromatography using silica gel as adsorbent and eluted with $CHCl_3/(CH_3)_2C{=}O/CH_3COOH$ (95:4:1, v/v/v). The glycerides were visualized by exposure to iodine vapor. To obtain pure triacylglycerol from the *n*-hexane layer, the triacylglycerols were separated on an alumina column eluting with a *n*-hexane/diethyl ether mixture (90:10, v/v). Free fatty acids from triacylglycerol obtained after saponification (*8*) were derivatized to their methyl esters and *N*-acyl pyrroldides following established procedure for gas–liquid chromatographic (GLC) and gas chromatographic−mass spectroscopic (GC-MS) analyses (*30*).

Formation of Fatty Acid Urea Inclusion Complex. The urea fatty acid complexation was accomplished following established procedure (*31*). Briefly, to the recovered free fatty acids (5 g) was added urea (20 g) in aqueous methanol (95%, 200 mL), and the urea fatty acid ratio was maintained at 4:1 (w/v) and 4 °C as established by our earlier experiment (*8*). The resulting PUFA concentrate obtained by urea complexation was dissolved in methanol, TBHQ (0.01% w/v) was added as antioxidant to increase the stability of the fatty acids, and the mixture was kept under a blanket of N_2 at −20 °C until further use.

Transmethylation of Free Fatty Acids and GC Analysis. Determination of the fatty acid composition in the released fatty acid fraction (by GLC) was carried out as described previously (*8*). Briefly, lipid from triacylglycerol was extracted by using $CHCl_3/CH_3OH/H_2O$ (2:4:1, v/v/v) following an established procedure (*32*), and the lipid extract thus obtained was saponified with 0.5 N KOH in CH_3OH. After removal of the nonsaponifiable material with *n*-hexane and acidification with 1 N HCl, the saponifiable materials were extracted with petroleum ether/diethyl ether (1:1, v/v) and transesterified to furnish fatty acid methyl esters (FAME) by reaction (30 min under reflux) with a methylating mixture (14% BF_3/MeOH) in a boiling water bath under an inert atmosphere of N_2 (*33*). The FAME thus obtained was extracted with *n*-hexane/water, and the solvent layer was concentrated, reconstituted in petroleum ether, and stored in deep freeze (−20 °C) until required for analyses. A Perkin-Elmer AutoSystem XL gas chromatograph equipped with a flame ionization detector (FID) analyzed the composition of fatty acids. The column used was an Elite-5 (crossbond 5% diphenyl−95% dimethyl polysiloxane) capillary column (30 m × 0.53 mm i.d., 0.50 μm film thickness, Supelco, Bellefonte, PA). The oven temperature was held at 110 °C for 1 min and then increased to 250 at 30 °C/min, at which it was held for 1.0 min, followed by an increase of 25 °C/min to 285 °C, at which it was held for 2.0 min, until all peaks had appeared (*8*). The injector and detector were held at 285 and 290 °C, respectively. Helium was used as carrier gas at 3.0 cm/s flow rate. The injection volume was 1 μL. FAMEs were identified by comparison of retention times with known standards (37 component FAME Mix, Supelco).

***N*-Acyl Pyrrolidide Derivatization for Determination of Position of Double Bonds and GC-MS Analysis.** The EI/GC-MS analyses were performed on a single-quadruple mass spectrometer (MD-800, Fisons) with an on-column injector set at 110 °C for confirmation of the fatty acids identification. FAMEs were derivatized to *N*-acyl pyrrolidides by condensation of fatty acid methyl ester with a mixture of pyrrolidine (1 mL) and acetic acid (0.1 mL) at 100 °C under reflux (2 h) for GC-MS analyses (*30*). The GC apparatus was equipped with a WCOT fused silica capillary column of high polarity (DB-5; 30 m × 0.25 mm i.d., 0.39 mm o.d., and 0.25 μm film thickness; Varian). The polymeric stationary phase was nonpolar (VF-5MS, 5% phenyl-substituted methylsiloxane). The carrier gas was ultrahigh-purity He (99.99% purity) with a constant flow rate of 1 mL/min. The injector and detector temperatures were maintained isothermal at 300 °C. The injection volume was 1 μL. Samples were injected in split (1:15) mode at 300 °C into the capillary column, similar to that used for the GC analyses, and the oven was identically programmed. Ion source and transfer line were kept at 300 °C. Mass spectra were analyzed using Varian Workstation (version 6.2) software.

Silver Ion Thin-Layer Chromatography ($AgNO_3$-TLC). The recovered FAMEs from urea fractionation were resolved by TLC (5 cm × 20 cm), precoated with silica gel, and impregnated with $AgNO_3$. Fifteen grams of silica gel G was mixed with 10% (w/v) solution of $AgNO_3$ (40 mL) in methanol/water (9:1, v/v) and spread in a uniform thickness (0.25 mm). Plates were drained, air-dried, activated at 110–120 °C in dim light for 30 min, and stored in a light-tight desiccator container. The methyl esters were applied to the plate as a narrow band. The plates were developed twice in *n*-hexane/diethyl ether/acetic acid (94:5:1, v/v/v) to separate individual bands. The bands were stained with 2,7-dichlorofluorescein in methanol (0.1%, w/v) and examined under UV-light.

Statistical Analyses. The percentage composition of individual fatty acid methyl esters was expressed as mean ± standard deviation of three different experiments and subjected to a one-way analysis of variance (ANOVA) using SPSS (ver. 13.0) software. Arc sin transformation was used prior to statistical analyses of FAME data expressed in percentages. On the basis of the significance of treatments, LSD at 5% level of significance ($p = 0.05$) was computed.

Table 1. Scheme of Lipase Purification from Crude Culture Broth of *B. licheniformis* MTCC 6824

purifn step	lipase specific activity[a] (LU/mg)	yield (%)	purifn factor
crude culture broth[b] (48 h, 37 °C)	2.50 ± 0.24	100	1
ammonium sulfate precipitation	10.66 ± 1.88	29.85	4.26
dialysis	29.81 ± 2.41	25.11	11.92
anion exchange chromatography[c]	83.82 ± 10.01	16.42	33.53
gel exclusion chromatography	520.28 ± 123.3	8.36	208.11

[a] The unit for specific activity is micromolar 4-nitrophenol released per minute per milligram of total protein. [b] Culture broth collected after incubation at 37 °C for 48 h. [c] Chromatographic purification of lipase was carried out on anion exchanger Amberlite IRA-410 (Cl^- form) and gel exclusion column (Sephadex G-100), respectively.

RESULTS AND DISCUSSION

GC-MS Analyses of FAMEs and *N*-Acyl Pyrrolidides. The molecular ions for SFA and MUFA-FAME were conspicuous as obvious from the mass spectra. An ion at $(M - 31)^+$ represents the loss of a $-OCH_3$ group, thus confirming the molecular structure as methyl ester. The base peak was assigned to be the 1-methoxyethenol moiety (*m*/*z* 74) obtained by McLafferty rearrangement. The spectra further contained lower *m*/*z* fragment ions at a difference of *m*/*z* 14 definitely supporting the general structure of FAME. The McLafferty ion was found to be less conspicuous in EI-MS of LC-PUFAs having more double bonds ($n > 4$), which undergoes complex rearrangement under high-energy EI conditions. Formation of cyclic tropylium ion (*m*/*z* 91) in fatty acids with four or more double bonds was apparent in PUFAs. The EI-MS spectrum of methyl linoleate has an abundant molecular ion (*m*/*z* 294) and base peak as the McLafferty ion (*m*/*z* 74). In methyl eicosapentaenoate and methyl docosahexaenoate, one fragment ion at *m*/*z* 108 defines an n-3 terminal group. In the FAME EI-MS fragmentation pattern of LC-PUFAs with more double bonds it is difficult to determine the location of the double bond from the spectra due to migration of the double bond along the long aliphatic chain. EI-MS of *N*-acyl pyrrolidides minimizes the migration due to higher stability of the pyrrolidine moiety. In *N*-acyl pyrrolidides, the base peak was found to be the McLafferty rearrangement ion at *m*/*z* 113. A uniform distribution of fragment peaks is apparent at every *m*/*z* 14 units except in the vicinity of the double bond, where the interval is *m*/*z* 12 units.

Purification of *B. licheniformis* MTCC 6824 Lipase. An extracellular lipase produced by *B. licheniformis* MTCC 6824 was purified by chemical precipitation and chromatography. The enzyme solution, obtained after precipitation with $(NH_4)_2SO_4$ and ethanol/ether, exhibited a specific activity of 4.84 mg/mL with a recovery of 29.85%. The lipase was purified 33.53-fold with 16.42% yield by anion exchange chromatography and 208.11-fold with 8.36% recovery by Sephadex G-100 gel exclusion chromatographic step (**Table 1**). High specific activity (520.28 LU/mg) of the purified lipase was apparent after the final chromatographic step. The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 74.8 kDa on SDS-PAGE gels (*13*).

Preparation of PUFA Concentrates from Sardine Oil by Lipase-Catalyzed Hydrolysis. The fatty acid composition of

Table 2. Percent Fatty Acid Composition of Crude and Lipase Hydrolysate of Sardine Oil at Three Different Times (1–9 h) Using Purified Lipase Obtained from *B. licheniformis* MTCC 6824 and PUFA Concentrates Obtained by the Urea Adduction at 4 °C and Urea/Fatty Acid Ratio of 4:1 (w/w)

		% composition of fatty acids in the lipase hydrolysate of sardine oil at different times				
fatty acid	CSO[a]	1 h	3 h	6 h	9 h	UAC[b]
saturated fatty acids						
12:0	0.06 ± 0.01	0.03 ± 0.00	ND	ND	ND	ND
14:0	7.04 ± 0.22	6.22 ± 0.19	5.19 ± 0.46	2.84 ± 0.57	2.55 ± 0.32	0.82 ± 0.18
16:0	0.45 ± 0.09	0.21 ± 0.05	0.11 ± 0.03	ND	ND	ND
17:0	0.28 ± 0.03	0.18 ± 0.03	0.08 ± 0.01	ND	ND	ND
Σ SFA	7.83	6.64	5.38	2.84	2.55	0.82
monounsaturated fatty acids						
16:1n-7 (Δ9)[c]	31.56 ± 2.59	29.17 ± 1.28	25.76 ± 2.14	21.64 ± 3.65	15.37 ± 1.16	7.28 ± 1.43
18:1n-9 (Δ9)	16.86 ± 1.18	15.53 ± 2.07	13.95 ± 1.68	9.32 ± 1.08	9.04 ± 0.93	3.72 ± 0.89
17:1 (Δ9)	0.59 ± 0.08	0.46 ± 0.06	0.31 ± 0.07	0.15 ± 0.03	0.23 ± 0.08	ND
20:1n-11(Δ9)	0.30 ± 0.14	0.25 ± 0.03	0.18 ± 0.04	0.11 ± 0.02	ND	ND
Σ MUFA	49.31	45.41	40.2	31.22	24.64	11
polyunsaturated fatty acids						
18:2n-6 (Δ9)	0.71 ± 0.23	0.62 ± 0.08	0.59 ± 0.11	0.41 ± 0.07	0.45 ± 0.13	1.98 ± 0.36
18:3n-3 (Δ9)	4.47 ± 0.84	4.32 ± 0.29	2.73 ± 0.86	2.38 ± 0.23	2.04 ± 1.27	5.62 ± 0.79
18:4n-3 (Δ6)	1.38 ± 0.35	1.29 ± 0.16	1.07 ± 0.31	0.68 ± 0.10	1.17 ± 0.22	3.73 ± 0.41
20:4n-6 (Δ5)	0.10 ± 0.02	0.55 ± 0.08	1.23 ± 0.33	3.94 ± 0.46	3.13 ± 0.57	5.80 ± 0.39
20:5n-3 (Δ5)	17.80 ± 1.57	27.14 ± 1.63	34.25 ± 1.92	46.29 ± 2.15	47.11 ± 2.07	55.38 ± 1.46
22:5n-3 (Δ7)	1.14 ± 0.08	1.09 ± 0.16	0.82 ± 0.18	0.75 ± 0.14	1.03 ± 0.19	1.97 ± 0.41
22:6n-3 (Δ4)	7.67 ± 1.50	7.47 ± 0.36	7.61 ± 0.43	7.02 ± 0.18	7.59 ± 0.43	9.04 ± 0.63
Σ PUFA	33.27	42.48	48.3	61.47	62.52	83.52
EPA/ARA	178.00	49.35	27.85	11.75	15.05	9.55
n-3/n-6	40.07	35.31	25.54	13.13	16.46	9.74
Δ5/Δ4-PUFA	2.33	3.71	4.66	7.16	6.62	6.77
Δ5/Δ6-PUFA	12.97	21.47	33.16	73.87	42.94	16.40
Δ5/Δ9-PUFA	3.46	5.61	10.69	18.00	20.18	8.05
LSD ($p = 0.05$)	0.95	1.28	1.05	2.36	0.82	2.49

[a] CSO, crude sardine oil. [b] UAC, urea concentrate of fatty acids obtained after lipase-catalyzed hydrolysis of sardine oil for up to 6 h. [c] Δ9 indicates fatty acids with Δ9-olefinic double bond; likewise, the numeric preceded by Δ reveals fatty acids with double bonds at that position; individual fatty acid is expressed as percentage of total identifiable fatty acids. ND, fatty acids identified on the GC trace, but not integrated by the instrument; ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids. Data are presented as mean values of three samples (mean ± standard deviation). On the basis of the significance of treatments, LSD at 5% level of significance ($p = 0.05$) was computed. At the beginning of the hydrolysis (3 h) the lipases display a significant preference for unsaturated fatty acids containing 14–18 carbon atoms. However, the resistance to release SFAs and MUFAs was less as the hydrolysis reaction progressed. These values do not total 100% because minor fatty acids are not reported.

sardine oil and those of enzyme hydrolysates are given in **Table 2**. Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), whereas 16:1n-7 contributed the major share (>31% TFA) among all of the individual fatty acids in the crude sardine oil (**Table 2**). EPA and DHA were found to be the major n-3 PUFAs, contributing 17.8 and 7.67% of TFA, respectively. The n-6 fatty acids have a minor share in the total fatty acid content of sardine oil (0.81% TFA).

The total PUFA content of triacylglycerol increased with time up to 6 h (57.47%), beyond which it remained relatively constant (59.5% after 9 h). The active sites of lipase execute the process of acylation through a charge relay system to produce an intermediate acyl-lipase binary complex (*34*). This binary complex of enzyme and saturated fatty acid containing triacylglycerol was hydrolyzed to furnish free lipase. Lipase-catalyzed hydrolysis resulted in an increase in the PUFA content as high as 72.73% after 6 h of hydrolysis, whereas MUFA and SFA levels showed reductions of 36.69 and 63.73%, respectively (**Table 2**).

n-3 Fatty Acids. The n-3 fatty acids exhibited an increase of 63.65% in the triacylglycerol after 6 h of lipase hydrolysis. In the case of EPA and DHA, there was a continuous increase in their concentration up to 6 h. However, their percent concentration decreased as reaction time increased beyond 6 h and ultimately reached a plateau (**Table 2**). The Δ5 n-3 PUFA EPA content in triacylglycerol mixture increased proportionally with the progress of hydrolysis. After 6 h, the EPA content in the glyceride mixture was 46.29%, and the overall EPA content of the released fatty acid fraction after 9 h was 47.11% compared with 17.8% in the original oil, that is, a 2.65-fold increase. The overall DHA composition of the released fatty acid fraction after 6 h of hydrolysis was 3.02% compared with 7.67% in the original oil, that is, a 2.54-fold decrease. The content of linolenic acid (18:3n-3) containing a Δ9-double bond was found to be reduced after enzymatic hydrolysis (2.04% after 9 h).

n-6 Fatty Acids. The n-6 fatty acids had a minor share in the total fatty acid content of sardine oil (0.81% TFA). The major n-6-PUFA, ARA, content in the glyceride mixture increased with the progress of hydrolysis. After 3 h, the ARA content in the glyceride mixture was 1.23%, and after 6 h, the value was 3.94%, which were substantially higher than that in the crude sardine oil (0.10%).

Monoenoic and Nonenoic Fatty Acids (MUFAs and SFAs). The reduction in SFAs and MUFAs in the enzyme hydrolysate appeared to be due to the higher selectivity of lipase for SFAs and MUFAs, thus furnishing a fatty acid concentrate with comparatively higher PUFA content (**Table 2**).

Fatty Acid Specificity of Lipases toward LC-PUFAs with a Δ5 Unsaturated Double Bond. The structure of fatty acids and the fatty acid compositions of sardine oil are shown in **Figure 1** and **Table 2**, respectively. The results indicated that the lipase is unique in its resistance to EPA and ARA. The hydrolysis reaction raised EPA and ARA concentrations by 2.60- and 34.4-fold, respectively, after 6 h. This could be attributed

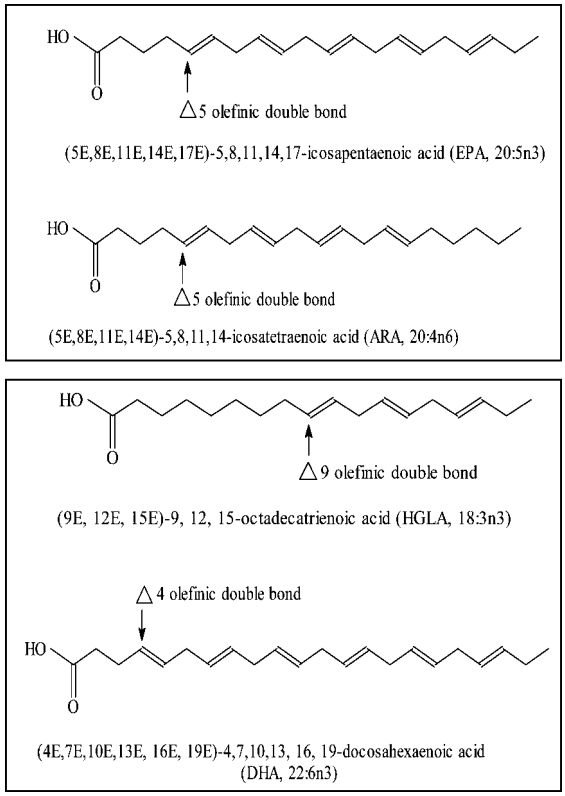

Figure 1. Chemical structures of the Δ5 and non-Δ5 class polyunsaturated fatty acids.

to the positional specificity of the enzyme to the olefinic double bonds of PUFAs. The decrease in the content of SFAs and MUFAs in the glyceride mixture with the progress of hydrolysis suggests that SFAs and MUFAs were more easily hydrolyzed by the lipase than those in triacylglycerol that contain EPA and ARA, resulting in their enrichment in the triacylglycerol fraction.

The results suggest the fatty acid triacylglycerols containing Δ6 and Δ9 isomers were more susceptible to lipolysis by the *B. licheniformis* MTCC 6824 lipase, and the discrimination was the greatest for the Δ5 isomer (EPA and ARA) followed by Δ4 double-bonded fatty acids such as DHA. Hayes and Kleiman (*35*) reported that lipases discriminated Δ5 fatty acids of meadowfoam oil to various degrees and that these lipases released Δ5 fatty acids quite slowly by hydrolysis reaction. The results of the present study on the specificity of lipase toward sardine oil containing different fatty acids, with various double bonds, namely, Δ9 (18:2n-6 and 18:3n-3 among PUFAs; and 18:1n-9, 16:1n-7, and 20:1n-11 among MUFAs), Δ6 (18:4n-3), Δ5 (20:5n-3 and 20:4n-6), Δ7 (22:5n-3), and Δ4 (22:6n-3) revealed that the lipase is less reactive with the Δ5 double bond among the PUFAs. Thus, by using the lipase, *cis*-Δ5 and non-*cis*-Δ5 PUFAs may be obtained from fats and oils containing other fatty acids (**Figure 2**). Such fatty acid selectivity of lipases, that is, hydrolysis favoring EPA over DHA and GLA or ARA over 18:4n-6, may therefore be useful to fractionate EPA and ARA from sardine oil.

Comparison of all the polyunsaturated fatty acids with EPA and ARA showed that all have olefinic double bonds but that the double bond is positioned at the number 5 carbon [*cis*-5 double bond (Δ5 position)] from the carboxyl end. In the case of lipase, regardless of the kind of fatty acid (n-3 or n-6) and the number of double bonds, EPA and DHA remained in the triacylglycerol fraction. Fatty acids other than EPA and ARA esterified on glycerides were selectively hydrolyzed by lipase. These results indicate that the lipase was preferentially reactive

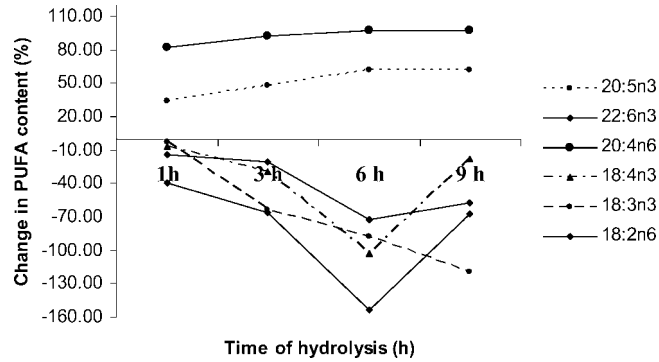

Figure 2. Percent change in the PUFA content in the triacylglycerol fraction obtained in the *n*-hexane layer during hydrolysis of PUFA-rich oils with *B. licheniformis* MTCC 6824 lipase.

for fatty acids other than Δ5-PUFAs. In the case of lipase, a higher proportion of Δ5-PUFAs was observed in the triacylglycerol fraction at all stages of hydrolysis, resulting in an increase of Δ5 unsaturated fatty acids as the degree of hydrolysis increased, suggesting the preferential hydrolysis of the fatty acids other than the Δ5 unsaturated bond.

EPA Enrichment by Urea Fractionation. The fatty acid compositions in the crude sardine oil, enzyme-catalyzed hydrolysate, and urea concentrates are depicted in **Table 2**. The fatty acids containing enriched EPA (46.29%) and ARA (3.94%) recovered by partial hydrolysis (6 h) using lipase were concentrated by urea adduction, so that the final EPA and ARA compositions were 55.38 and 5.8%, respectively, and the total percentage of PUFA was 79.56%. The percentage of EPA (55.38%) substantially increased after urea fractionation as compared to that of DHA (5.08%) (**Table 2**). The fractionation results show a total reduction in the content of saturated fatty acids (14:0, 16:0, and 18:0), a large reduction in MUFAs, and a remarkable increase in PUFAs. A total of 71.78% of the total fatty acids in the concentrate were n-3 PUFAs. EPA was enriched from 46.29 to 55.38% and DHA from 4.02 to 5.08%. The level of 16:1n-7, the predominant MUFA, was reduced by 66.36% by urea fractionation. These results suggest that the urea adduction method could be very useful for the enrichment of all PUFAs irrespective of the position of olefinic double bonds.

$AgNO_3$-TLC. $AgNO_3$-TLC was used to determine the progress of purification of PUFAs after urea complexation. The results were confirmed by GC/GC-MS to determine the fatty acids profile. The uppermost band (R_f, 0.79) of saturated fatty acids and the second band of monoenoic fatty acids (R_f, 0.45) were apparent, and the results are consistent with our earlier studies (*8*). Tetraene, pentaene, and hexaene methyl esters appeared to be concentrated in the lower band of the TLC plates (R_f, 0.15–0.30). EPA, ARA, and DHA FAMEs were apparent at the base of the TLC chromatogram (R_f, 0.22–0.25).

In conclusion, the present study showed that *B. licheniformis* MTCC 6824 lipase is a potential enzyme source for the concentration of n-3 and n-6 PUFAs, particularly Δ5 olefinic double-bond fatty acids, namely, EPA and ARA. The combination of lipase-catalyzed hydrolysis and urea complexation is a promising method to obtain n-3 PUFA concentrates from sardine oil. The lipase-catalyzed reaction offers several benefits such as greater selectivity and milder reaction conditions as compared to that of chemical hydrolysis, which yields undesirable oxidation products and polymers. Further optimization of hydrolysis parameters will be taken up in future studies for obtaining higher

recovery of targeted PUFAs from different marine sources, which will pave the way for lipase application on a commercial scale.

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