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Selective enrichment of *n*−3 polyunsaturated fatty acids with C_{18}–C_{20} acyl chain length from sardine oil using *Pseudomonas fluorescens* MTCC 2421 lipase

Kajal Chakraborty *, R. Paul Raj

Physiology Nutrition and Pathology Division, Central Marine Fisheries Research Institute, Ernakulam North P.O., P.B. No. 1603, Cochin 682 018, Kerala, India

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ABSTRACT

An extracellular lipase purified from *Pseudomonas fluorescens* MTCC 2421 was used to enrich sardine oil triglycerides with eicosapentaenoic acid (20:5 *n*−3) and linolenic acid (18:3 *n*−3) to 35.28% and 8.25%, respectively, after 6 h of hydrolysis. The corresponding *n*−6 fatty acids (18:2 *n*−6 and 20:4 *n*−6) exhibit a reduction (54.93% and 50%, respectively). Structure–bioactivity relationship analyses revealed that the lower hydrophobic (log*P* values) constants of 18:3 *n*−3 and 20:5 *n*−3 (5.65 and 5.85, respectively) result in their higher hydrolytic resistance towards lipase, leading to their enrichment in the triglyceride fraction after lipase-catalysed hydrolysis. Lipase-catalysed hydrolysis of sardine oil for 6 h followed by urea fractionation at 4 °C with methanol provided free fatty acids containing 42.50% 20:5 *n*−3 and 10.31% 18:3 *n*−3, respectively. Argentation neutral alumina column chromatography, using *n*-hexane/ethylacetate (2:1, v/v) resulted in 20:5 *n*−3 of high purity (83.62%), while 18:3 *n*−3 was found to be eluted with *n*-hexane/dichloromethane (4:1, v/v) as eluting solvent with a final purity of 75.31%.

1. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs), viz., eicosapentaenoic acid (EPA, 20:5 *n*−3), docosahexaenoic acid (DHA, 22:6 *n*−3) and linolenic acid (LA, 18:3 *n*−3) are widely available in a large variety of marine organisms, like microalgae, polychaetes, fin fish and shellfish. These LC-PUFAs are recognised to have special pharmacological and physiological effects on human/animal health (Harris, 1989). Marine fish larvae and broodstock lack the adequate titre of the essential enzymes (like elongase and desaturase) required to synthesise polyunsaturated fatty acids (PUFAs) *de novo* in sufficient quantity from precursor molecules like short-chain fatty acids. Therefore they require greater concentrations of PUFAs for their growth, reproduction and survival (Cahu, Guillaume, Stephan, & Chim, 1994). Diets deficient in these PUFAs have been found to have a negative effect on ovarian development, fecundity and egg quality (Harrison, 1990). Since these physiological functions drew attention to these essential fatty acids, the production of PUFA-rich fish oil as a food material is a growing research area. For commercial exploitation of a high value added products such as PUFA concentrates, the acids must first be separated from their triglycerides and purified to a high degree based on differences in physico-chemical properties associated with the number of double bonds in the molecule or acyl chain length (Chakraborty & Paulraj, 2007). Most of the existing chemical purification methods are based on hydrolysis of oils to free fatty acids; these methods are non-selective to different fatty acids. Lipases (triacylglycerol acyl hydrolases) are one of the most important classes of hydrolytic enzymes that specifically hydrolyse carboxyl esters of triglycerides into free fatty acids and partial acylglycerols. Lipases have been used as animal feed supplement to increase bioavailability of PUFAs (Akoh, Jennings, & Lillard, 1996; Huang & Akoh, 1994; Kosugi & Azuma, 1994). Chemical hydrolysis may partially destroy the natural all *cis*-PUFAs if the process is inadequately carried out due to the high temperatures involved (Heimermann, Holman, Gordon, Kowalyshyn, & Jensen, 1973). The mild conditions used in enzymatic reactions offer a promising alternative to chemical hydrolysis, avoiding the formation of undesirable oxidation products, polymers, and isomeric conversion of natural all *cis*-PUFAs to deleterious *trans*-PUFAs. The unique characteristics of lipases, i.e., substrate, positional (acyl side-chain and olefinic double bonds) and stereospecificity can be utilised to selectively concentrate targeted fatty acids in triglycerides that can be readily absorbed into plasma triglycerides (Jaeger & Reetz, 1998). Many attempts have been carried out in trans- or interesterification reactions to modify and enrich the content of PUFA in triglycerides by using various lipases as biocatalysts (Hoshino, Yamane, & Shimizu, 1990; Sridhar & Lakshminarayana, 1992).

Lipases occur widely in animals, plants and microorganisms (Jaeger & Eggert, 2002). Among microbial lipases bacterial lipases are the most widely used class of enzymes in biotechnological applications because of their higher stability (Chakraborty &

* Corresponding author. Tel.: +91 484 2394867/4069936, +09447084867; fax: +91 0484 2394909.
E-mail address: kajal_iari@yahoo.com (K. Chakraborty).

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Paulraj, 2008a; Ternstrom, Lindberg, & Molin, 1993). The unique substrate specificity of microbial lipases has been utilised for the enhancement of PUFA content in fish oils by several groups (Harris, 1989; Matori, Asahara, & Ota, 1991). Isolates of *Pseudomonas fluorescens* have been found to produce enzymes active on lipolytic substrates under alkaline conditions (Kojima & Shimizu, 2003). Lipase genes from *Pseudomonas* have been cloned and expressed in *Escherichia coli*, due to their potential industrial applications (Kojima, Kobayashi, & Shimizu, 2003). *P. fluorescens* SIK W1 was found to produce extremely heat-stable lipase that has high lipolytic activity for short- to medium-chain triacylglycerols (Chung, Lee, Yoo, & Rhee, 1991). An extracellular alkaline metallolipase with molecular weight of 74.8 kDa derived from cultures of *Bacillus licheniformis* MTCC 6824 was found to enrich Δ5 olefinic double bond fatty acids, viz., EPA and AA (Chakraborty & Paulraj, 2008b). A thermophilic and alkalophilic lipase from *Bacillus coagulans* BTS-3 was purified and biochemically characterised (Kumar, Kikon, Upadhyay, Kanwar, & Gupta, 2005). A lipase produced by recombinant *B. licheniformis* was found to be stable at alkaline pH of 12.0 (Nthangeni, Patterton, van Tonder, Verger, & Litthauer, 2001). These results are in contrast to thermotolerant lipases from *Bacillus thermoacetenulatus* and *Bacillus thermoleovorans*, which display maximum activity at pH 8.0 (Lee et al., 1999; Rua, Schmidt-Dannert, Wahl, Spraner, & Schmidt, 1997). *B. coagulans* NCIMB 9365 has been reported to possess an intracellular carboxylesterase (Molinari, Brenna, Valenti, & Aragozzini, 1996). The substrate specificity of lipase has been utilised for the recovery of EPA (Δ5) and DHA (Δ4) from marine oils and γ-linolenic acid (Δ6) from borage seed oil (Morioka, Maeda, & Ishida, 1987). DHA-rich triglycerides were prepared from fish oil with lipases obtained from *Candida cylindracea* and *Chromobacterium viscosum* (Tanaka, Hirano, & Funada, 1992, 1994). Substrates containing Δ2–Δ7 isomers of 18:1 were resistant to pancreatic lipase-catalysed hydrolysis, resulting in higher concentrations of oleic acid, and the discrimination was the greatest for the Δ5 isomer (Heimermann et al., 1973).

PUFAs are widely available in a large variety of marine organisms, but sardine oil is easily available, cheap and contains considerable amount of PUFAs (33.26%) particularly EPA (>15%). The present paper is directed to a process for preparing long-chain PUFA concentrates particularly *n*−3 PUFAs with C_{18}–C_{20} acyl chain length, by lipase-catalysed purification of triglycerides derived from sardine oil, and further concentrating the PUFAs by physical and chromatographic methods. Structure–activity relationship analysis of different classes of fatty acids and microbial lipase can be used as a tool to elucidate the structural descriptors of fatty acids controlling lipolytic activity. However, no focused studies have been reported in the literature to correlate the hydrolytic activity of lipase, *vis-à-vis* the structural parameters of fatty acids responsible for bioactivity. In this study we report the structure–bioactivity correlation analyses of fatty acids and *P. fluorescens* MTCC 2421 lipase by utilising hydrophobic and steric descriptors to observe the variability in the olefinic and alkyl side-chain of fatty acids and their effect on lipase hydrolytic activity.

2. Materials and methods

2.1. Chemicals and reagents

All chemical reagents, viz., methanol, *n*-hexane, NaOH, urea, and molecular sieve were obtained from Qualigens (Mumbai, India). The solvents were of analytical grade (Merck, Darmstadt, Germany), and were redistilled in an all-glass system. Sephadex G-100 and other supports of chromatography were from Sisco Research Laboratories (SRL, Mumbai, India). All glassware was rinsed with $CHCl_3/CH_3OH$ (2:l, v/v), and dried under N_2. Standards of fatty acid methyl esters (Supelco TM 37 Component FAME Mix, Catalogue No. 47885-U) and boron trifluoride/methanol (14% BF_3 in CH_3OH, 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, and stabilised with butylated hydroxyquinone (0.01% w/v). The oil was stored under nitrogen at −20 °C, in a sealed dark amber glass container, until use.

2.2. Preparation of C_{18}–C_{20} polyunsaturated fatty acid concentrates by *P. fluorescens* MTCC 2421 lipase-catalysed hydrolysis of sardine oil triglycerides

Triglycerides from refined sardine oil, as extracted by an established method (Bligh & Dyer, 1959) were further purified on a column of neutral alumina using *n*-hexane/diethyl ether as eluting solvent system (95/5, v/v). The triglyceride obtained after chromatographic purification was evaporated *in vacuo*.

An extracellular lipase from 48 h broth culture of *P. fluorescens* MTCC 2421 was purified to homogeneity by a combination of ammonium sulphate precipitation (70% saturation) and chromatographic separation on anion exchanger Amberlite IRA 410 (Cl^- form) and Sephadex G-100 gel exclusion chromatography. The lipase-catalysed hydrolysis of triglycerides from refined sardine oil was performed following established procedure (Tanaka et al., 1992) with modification. To the reaction mixture of triglycerides (100 ml, 0.01% w/w *tert*-butylhydroquinone) in PIPES–NaOH buffer (5 ml of 0.1 M solution at pH 7.0) stabilised with Triton X-100 (0.5% v/v), and $CaCl_2$ (0.4 ml of 100 mM), purified lipase (*P. fluorescens* MTCC 2421 lipase, 500 LU) was added in a 250 ml-screw cap round-bottomed flask, to initiate the hydrolysis. The reaction flask was flushed with N_2 to replace air and to prevent oxidation, and placed into an incubator at 45 ± 1 °C with stirring (500 rpm). Samples (0.5 ml) from the reaction mixture were withdrawn periodically (3–9 h), and methanolic KOH (0.5 N, 25 ml) solution was added to the mixture to neutralise the free fatty acids released during hydrolysis, and the triglycerides were extracted thrice with *n*-hexane (3 × 100 ml). Hydrochloric acid (2 N HCl, 10 ml) was added to the mixture to neutralise the alkali, and the fatty acids in the aqueous layer were extracted with *n*-hexane (2 × 100 ml). The lower aqueous layer was discarded, and the upper *n*-hexane layer containing triglycerides was further extracted with distilled water (3 × 50 ml), to remove free fatty acids. The *n*-hexane layer was vacuum concentrated at 40 °C, using a rotary evaporator. The concentrated triglycerides were maintained under N_2 at −20 °C until further use. The hydrolysis products of the reaction catalysed by lipase were monitored by thin-layer chromatography (TLC), using silica gel as adsorbent, and eluted with chloroform/acetone/acetic acid (95:4:1, v/v/v). The glycerides were visualised by exposure to iodine vapour. To obtain pure triglycerides from the *n*-hexane layer, the triglycerides were separated on a neutral alumina column eluting with *n*-hexane/diethyl ether mixture (95/5, v/v). Free fatty acids obtained from triglycerides after saponification (Metcalf, Schimtz, & Pleka, 1966) were derivatised to their methyl esters and *N*-acylpyrrolidides, using an established procedure for gas chromatographic (GC) and gas chromatographic–mass spectroscopic (GC–MS) analyses (Andersson, 1978).

2.3. Purification of PUFAs by urea complexation

The triglycerides were saponified following an established procedure (Chakraborty & Paulraj, 2007) to furnish free fatty acids, to which (3 g) urea (12 g) in aqueous methanol (120 ml) was added, at the urea:fatty acid ratio of 4:1 (w/v); the contents were heated (60–65 °C) until a clear homogeneous solution was obtained. The urea fatty acid complexation was accomplished following an estab-

lished procedure with modification (Guil-Guerrero, Campra-Madrid, & Belarbi, 2000). The urea complexes were allowed to crystallise overnight at 4 °C. The mixture was then filtered, after which the methanolic solution of non-urea-complexed fatty acids was evaporated and poured into 5 ml of 1% HCl. The mixture was further extracted twice with 5 ml of *n*-hexane, and then the combined organic layers were washed with water, and dried over anhydrous sodium sulphate, before being evaporated. The resulting PUFA concentrate obtained by urea complexation was dissolved in methanol, and trans-esterified to furnish the FAMEs, which were extracted with *n*-hexane, and concentrated under reduced pressure in a blanket of N_2, to yield a residue. The residue was dissolved in *n*-hexane (30 ml) to be applied on an argentation chromatography column for analysis by GC/GC–MS as described previously (Chakraborty & Paulraj, 2007).

2.4. Argentation chromatographic fractionation of individual PUFAs

An aliquot of the concentrate was subjected to vacuum liquid chromatography over argentated neutral alumina (70–230 mesh), and the cold-water jacketed column was eluted with an *n*-hexane/diethyl ether step gradient increasing the proportion of diethyl ether (9.5:0.5 to 1:1, v/v) to provide eight fractions (F_I–F_{VIII}, 50 ml). Hexane was used to remove the non-polar fraction followed by diethyl ether to give the polar fraction. Fraction F_V as eluted with *n*-hexane/diethyl ether (7.5:2.5, v/v) was further fractionated by column chromatography over neutral alumina using *n*-hexane/CH_2Cl_2 (95:5, 90:10, 80:20, and 75:25, v/v) as eluants to furnish 20 sub-fractions (SF_I–SF_{XX}). TLC-guided combination of the sub-fractions SF_{VII}–SF_{XIV} furnished linolenic acid of high purity (96.3%). The fraction F_{VI} (using *n*-hexane/diethyl ether, 3:2, v/v) was further separated by argentation column chromatography on neutral alumina, using a stepwise gradient system (*n*-hexane to ethyl acetate). EPA was obtained using *n*-hexane/ethyl acetate (2:1, v/v) as the eluting solvent system. Repeated elution, where necessary, was used to purify the fatty acid. Evaporation of solvents from fractions followed by TLC over silver-ion thin-layer chromatography ($AgNO_3$/TLC, 5 cm × 20 cm) (Chakraborty & Paulraj, 2007) using *n*-hexane/diethyl ether/acetic acid (94:5:1, v/v/v) validated the purity of the individual fatty acids. The TLC bands were stained with 2,7-dichlorofluorescein in methanol (0.1%, w/v), and examined under UV light.

2.5. Derivatisation of fatty acids for gas chromatography (GC)/gas chromatography–mass spectrometry (GC–MS) analyses

The fatty acid composition of the PUFA concentrate was measured as described previously (Chakraborty & Paulraj, 2007). In brief, triglycerides were extracted by using $CHCl_3/CH_3OH/H_2O$ (2:4:1, v/v/v), and saponified with alkaline reagent (0.5 N KOH/CH_3OH). The saponifiable materials were extracted with petroleum ether: diethyl ether (1:1, v/v) after removal of nonsaponifiable materials (terpenes, steroids, etc.) by solvent extraction (with *n*-hexane) and acidification (1 N HCl). Trans-esterification of the saponifiable materials was accomplished by reaction (30 min under reflux) with a methylating mixture (14% BF_3/CH_3OH) in a boiling water bath under an inert atmosphere of N_2 to furnish fatty acid methyl esters (FAME), which were extracted with *n*-hexane/water. After removal of the aqueous layer, the *n*-hexane layer was concentrated *in vacuo*, reconstituted in petroleum ether, and stored at −20 °C until required for analyses. A Perkin–Elmer Auto-System XL gas chromatograph (Perkin–Elmer, Waltham, MA) equipped with a flame ionisation detector (FID) was used to analyse the composition of the fatty acids. The column used was an Elite-5 (cross-bond 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 °C at 30 °C/min, where it was held for 1.0 min, followed by an increase of 25 °C/min to 285 °C, where it was held for 2.0 min, until all peaks had appeared. The injector and detector were held at 285 and 290 °C, respectively. Helium was used as carrier gas at 3.0 cm/s linear velocity. The injection volume was 1 μl. FAMEs were identified by comparison of retention times with known standards (37 component FAME Mix, Supelco).

The GC–MS analyses were performed on a single quadrupole mass spectrometer (Varian 1200L, Varian Inc., Palo Alto, CA) under electron impact (EI, ionisation energy 70 eV) conditions with an on-column injector set at 110 °C for confirmation of the identities of the fatty acids. FAMEs were derivatised to *N*-acylpyrrolidides 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, as reported earlier (Andersson, 1978). The GC was equipped with a VF-SMS fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Varian). The carrier gas was helium (99.99% purity) with a constant flow rate of 1 ml/min. The injector and detector temperatures were maintained at 300 °C. The injection volume was 1 μl. Samples were injected in split (1:15) mode using the same temperature programme as that used for the GC analyses. Ion source and transfer line were kept at 300 °C. Mass spectra were analysed using Varian Workstation (version 6.2) software.

2.6. Structure–bioactivity correlation analyses of fatty acids and P. fluorescens MTCC 2421 lipase

Structure–bioactivity relationship analysis is a useful tool in elucidating which essential structural features of different classes of fatty acids govern the enzyme (lipase) activity towards the esteric bonds of fatty acids. Structure–bioactivity relationship analyses utilised the physico-chemical parameters of biomolecules in a congeneric *n*−3 and *n*−6 fatty acid series for predicting their susceptibility or resistance towards lipolytic activity. To observe the variability in the olefinic and alkyl side-chain, hydrophobic and steric parameters were used. The hydrophobic descriptors, viz., partition coefficient (log*P*) and steric descriptors, viz., parachor (*P*) and molecular volume (MV) were generated using ACD Chemsketch (version 2) software (Chakraborty & Devakumar, 2005; Chakraborty & Devakumar, 2006). Enthalpic effects of the fatty acid molecule were calculated using the hydrophobic parameter (log*P*), which was derived from the partition coefficient.

2.7. Statistical analyses

Percentage composition individual fatty acid methyl esters were subjected to a one-way analysis of variance using SPSS (version 10.0) software. All measurements were performed in triplicate, and the data presented as mean values of three samples (mean ± standard deviation). Arc sin transformation was used prior to statistical analyses of FAME data expressed in percentages. Based upon the significance of treatments, LSD at the 5% level of significance ($p = 0.05$) was computed.

3. Results and discussion

3.1. Change in fatty acid composition as a function of time course (3–9 h) lipase-catalysed hydrolysis of triglycerides from sardine oil

The refined sardine oil was found to contain long-chain polyunsaturated fatty acids (LC-PUFAs), particularly eicosapentaenoic acid (20:5 *n*−3 or EPA; 17.80 ± 1.57% of total fatty acids, TFA)

and docosahexaenoic acid (22:6 *n*−3 or DHA; 7.67 ± 1.50% of TFA) along with other *n*−3 and *n*−6 PUFAs like linolenic acid (LA or 18:3 *n*−3; 4.47 ± 0.84% TFA), linolenic acid (18:2 *n*−6; 0.71 ± 0.23% TFA), and docosapentaenoic acid (DPA or 22:5 *n*−3; 1.14 ± 0.08% TFA) (Table 1). The *n*−6 fatty acids have a minor share of the total fatty acid content of sardine oil (0.81% TFA). The PUFAs containing C_{18}–C_{20} acyl chain length contributed a major share of the total fatty acids of the sardine oil (24.46% TFA). Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04 ± 0.22% TFA), while 16:1 *n*−7 contributed the major share (31.56 ± 2.59% TFA) among monounsaturated fatty acids (MUFAs) (Table 1). The sardine oil triglycerides were hydrolysed with lipases purified from *P. fluorescens* MTCC 2421, and the total fatty acid content of triglycerides at various time intervals of hydrolysis (3–9 h) were analysed, and the results are indicated below.

3.1.1. Saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs)

SFA levels showed a reduction of 16.48% and 37.42% after 3 and 6 h of hydrolysis, respectively, whereas after prolonged hydrolysis (9 h), the rate of reduction was recorded to be 35.63% (Table 1). The total MUFA content was found to be reduced by 11.01% after 3 h of lipase-catalysed hydrolysis. However, this group of fatty acids exhibited a reduction of 35.08% and 30.83%, after 6 and 9 h of hydrolysis, respectively. The decrease in the content of SFAs and MUFAs in the triglyceride mixture with the progress of hydrolysis suggests that SFAs and MUFAs were more easily hydrolysed by the lipase than those in triglycerides that contain EPA and LA, resulting in the enrichment of the latter in the triglyceride fraction (Fig. 1). An earlier report stated that substrates containing Δ2–Δ7 isomers of 18:1 were resistant to pancreatic lipase-catalysed hydrolysis resulting in higher concentration of oleic acid (18:1 *n*−9), and the discrimination was the greatest for the Δ5 isomer (Heimermann et al., 1973).

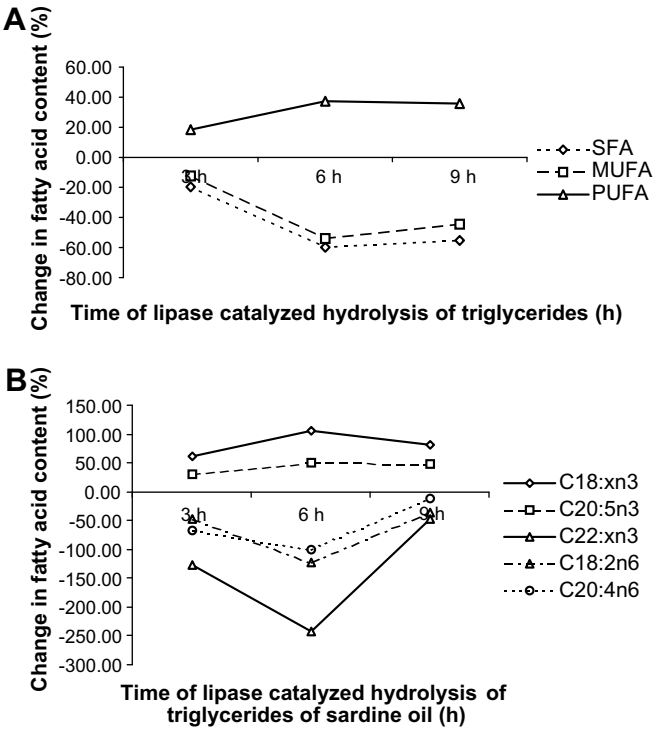


Fig. 1. Percent change in the fatty acids content in triglyceride fraction by *P. fluorescens* MTCC 2421 lipase-catalyzed hydrolysis of sardine oil for different time intervals (3–9 h). (A) Percent change in fatty acid content (SFAs, MUFAs, and PUFAs) in sardine oil triglycerides with respect to time duration of lipase-catalysed hydrolysis and (B) percent change in *n*−3 and *n*−6 fatty acid content with C_{18}–C_{22} acyl chain length in sardine oil triglycerides with respect to time duration of lipase-catalysed hydrolysis. C_{18}:*x*n−3 signifies sum total contribution of 18:3 *n*−3 and 18:4 *n*−3; C22:*x*n−3 signifies sum total contribution of 22:5 *n*−3 and 22:6 *n*−3.

Table 1
Fatty acid composition of crude and lipase hydrolysate of sardine oil at three different time duration (3, 6, and 9 h) using purified lipase obtained from *P. fluorescens* MTCC 2421 followed by urea complexation

Fatty acids	Sardine oil	% Fatty acid			
		3 h	6 h	9 h	UA–fatty acid[a]
Saturated fatty acids					
12:0	0.06 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	7.04 ± 0.22	6.15 ± 0.39	4.75 ± 0.23	4.92 ± 0.83	0.46 ± 0.08
16:0	0.45 ± 0.09	0.39 ± 0.03	0.15 ± 0.03	0.12 ± 0.02	0.03 ± 0.00
17:0	0.28 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
ΣSFA	7.83	6.54	4.90	5.04	0.49
Monounsaturated fatty acids					
16:1 *n*−7	31.56 ± 2.59	28.11 ± 2.16	18.33 ± 2.14	16.2 ± 1.98	8.15 ± 0.83
18:1 *n*−9	16.86 ± 1.18	15.28 ± 1.89	13.5 ± 1.76	17.73 ± 2.34	11.38 ± 1.05
17:1	0.59 ± 0.08	0.41 ± 0.05	0.18 ± 0.02	0.18 ± 0.03	0.00 ± 0.00
20:1 *n*−11	0.30 ± 0.14	0.08 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
ΣMUFA	49.31	43.88	32.01	34.11	19.53
Polyunsaturated fatty acids					
18:2 *n*−6	0.71 ± 0.23	0.48 ± 0.09	0.32 ± 0.06	0.52 ± 0.09	1.57 ± 0.21
18:3 *n*−3	4.47 ± 0.84	6.18 ± 0.41	8.25 ± 0.61	7.20 ± 0.55	10.31 ± 0.60
18:4 *n*−3	1.38 ± 0.35	2.09 ± 0.20	3.47 ± 0.16	2.42 ± 0.19	5.33 ± 0.49
20:4 *n*−6	0.10 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.09 ± 0.01	0.83 ± 0.18
20:5 *n*−3	17.80 ± 1.57	25.17 ± 3.04	35.28 ± 4.72	33.71 ± 5.01	42.50 ± 7.15
22:5 *n*−3	1.14 ± 0.08	0.56 ± 0.12	0.38 ± 0.07	0.83 ± 0.14	1.49 ± 0.23
22:6 *n*−3	7.67 ± 1.50	6.20 ± 0.33	5.35 ± 0.26	6.94 ± 0.95	8.22 ± 1.16
ΣPUFA	33.27	40.74	53.10	51.71	70.25
EPA/LA	3.98	4.07	4.28	4.68	4.12
n−3 PUFA	32.46	40.20	52.73	51.10	67.85
n−6 PUFA	0.81	0.54	0.37	0.61	2.40
n−3/*n*−6	180.33	74.44	142.51	83.77	28.27
LSD ($p = 0.05$)	0.95	1.17	1.39	2.17	1.98

[a] UA–fatty acid concentrate implies the urea concentrate of fatty acids. ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; and ΣPUFA: total polyunsaturated fatty acids. Data presented as mean values of three samples (mean ± standard deviation). These values do not total 100% because minor fatty acids are not reported.

3.1.2. The n−3 polyunsaturated fatty acids (PUFAs)

The variations of PUFA content of sardine oil triglycerides as a function of time during the lipase-catalysed hydrolysis are illustrated in Fig. 1A. The total *n*−3 PUFA of triglycerides increases with time up to 6 h of lipase-catalysed hydrolysis (52.73% TFA), beyond which it slowly decreased (51.10% TFA after 9 h) (Table 1). Among *n*−3 PUFAs, the content of 18:3 *n*−3 in the triglyceride mixture was recorded to be 6.18 ± 0.41% TFA after 3 h of lipase-catalysed hydrolysis, and after 6 h, the value was 8.25 ± 0.61% TFA, which was substantially higher than that in the crude sardine oil (4.47 ± 0.84% TFA). Similarly, the EPA content in triglyceride was found to increase by 41.40% after 3 h of hydrolysis, and 98.20% after 6 h (Fig. 1B). Attempts have been made by various workers to modify and enrich the content of individual PUFAs in triglycerides by using various lipases as biocatalysts by trans- or inter-esterification reactions. Several groups have utilised the unique substrate specificity of microbial lipases for the enhancement of PUFA content in fish oils. An earlier report described a lipase purified from *P. fluorescens* HU380 used to concentrate EPA and DHA from oils (Kojima & Shimizu, 2003). Immobilised lipases IM60 from *Mucor miehei* and SP435 from *Candida antarctica* were used to modify fatty acid composition of vegetable oils by enrichment of *n*−3 PUFAs (Huang & Akoh, 1994). In the present study, the C_{18}–C_{20} acyl chain lengthened *n*−3 PUFAs (EPA and LA) in the triglyceride mixture were found to increase proportionally (1.98-fold increase) with the progress of hydrolysis up to 6 h, after that their concentration plateaued, apparently due to the reduced selectivity of the lipase (Table 1). However, with the increase of acyl chain length (>C_{20}), the hydrolytic susceptibility of the ester linkage of triglycerides towards the lipase was found to increase as evident from the 1.43-fold decrease of DHA in the triglyceride fraction after 6 h of hydrolysis. In the present study, the microbial lipase was found to be specific towards hydrolysing *n*−6 fatty acids and *n*−3 fatty acids with >C_{20} acyl chain length (like docosapentaenoic acid and docosahexaenoic acid) at the initial stage of hydrolysis (up to 6 h) of fatty acyl ester bonds. The results also suggest that the esteritic bonds of C_{18}–C_{20} acyl chain lengthened *n*−3 PUFAs are resistant to hydrolysis by the lipase. However, after prolonged hydrolysis (>9 h), when only a few target fatty acid ester bonds (*n*−6 fatty acyl ester bonds and esters other than C_{18}–C_{20} *n*−3 fatty acids) are available in the enzyme hydrolysate that are susceptible to hydrolysis by a lipase, the microbial lipase can even cleave bonds that are resistant (or nearly resistant) to hydrolysis, i.e., EPA and LA (C_{18}–C_{20} *n*−3 fatty acids). These results indicate that the resistance of hydrolysis of this later class of fatty acyl ester bonds depends upon the presence of other substrates (*n*−6 fatty acyl ester bonds and esters other than C_{18}–C_{20} *n*−3 fatty acids) in the reaction mixture. It can be concluded that it might be possible to separate and concentrate C_{18}–C_{20} PUFAs with *n*−3 double bonds like EPA and LA using lipase from *P. fluorescens* MTCC 2421.

There are reports of modification of borage oil (*Borago officinalis* L.) fatty acid composition to incorporate EPA (31% EPA), with an immobilised SP435 lipase from *C. antarctica* as biocatalyst. With the progress of incubation time, EPA incorporation was also increased up to 36 h (Akoh & Sista, 1995). In an earlier attempt to concentrate the content of DHA in a glyceride mixture, fish oil was hydrolysed with six kinds of microbial lipase. When the hydrolysis with *C. cylindracea* lipase was 70% complete, the EPA content was recorded to be 70% of the original fish oil (Tanaka et al., 1992). DHA and EPA concentration was reported to be doubled by *Pseudomonas cepacia* and *Candida rugosa* lipase-assisted hydrolysis of Atlantic salmon (*Salmo salar* L.) viscera oil (Sun, Pigott, & Herwig, 2002). Six commercial lipases (Novozyme 435 from *C. antarctica*, Lipozyme IM from *M. miehei*, PS-30 from *Pseudomonas* sp., AP-12 from *Aspergillus niger*, AY-30 from *C. rugosa*, and Novozyme-677BG from *Thermomyces lanuginosus*) were tested for their ability to incorporate DHA into evening primrose oil by Senanayake and Shahidi (2004). Among the enzymes examined, Novozyme 435 from *C. antarctica* was chosen over the other enzymes to catalyse the trans-esterification reaction owing to higher

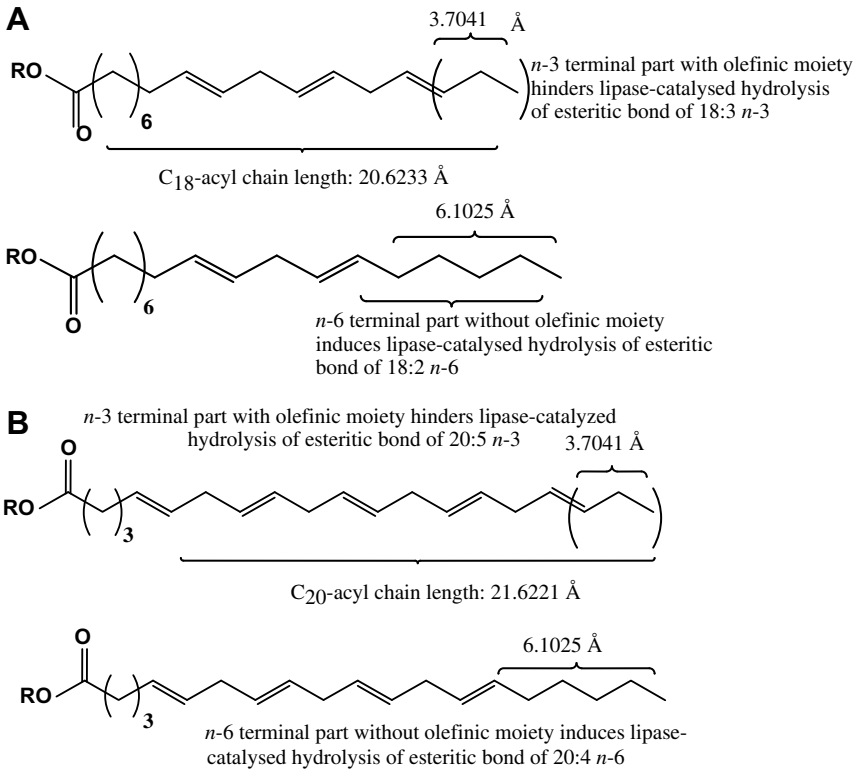


Fig. 2. Molecular sketch model of congeneric *n*−3 and *n*−6 polyunsaturated fatty acids. (A) C_{18} acyl chain length PUFAs with *n*−3 and *n*−6 moiety and (B) molecular sketch model of 20:5 *n*−3 and 20:4 *n*−6.

incorporation of DHA. For the time course reaction, incorporation of DHA increased up to 25.2% after 24 h (Senanayake & Shahidi, 2004). Synthesis of triglycerides by enzymatic esterification of PUFAs with glycerol, using microbial lipase Novozyme 435 (Novo Nordisk, A/S) from *C. antarctica*, was carried out to obtain a triglyceride yield of 93.5% from cod liver oil PUFA concentrate; the product was reported to contain 25.7% EPA and 44.7% DHA (Medina et al., 1999). DHA was enriched from the recovered free fatty acid fraction from sardine oil by selective enzymatic esterification using Lipozyme™ lipases that allowed up to 80% DHA enrichment but gave no EPA enrichment (Schmitt-Rozieres, Deyris, & Comeau, 2000). An earlier study reported the hydrolysis and urea adduction of refined cod oil including 12.2% EPA and 6.9% DHA with HU-lipase produced by *P. fluorescens* HU380 to furnish free fatty acids with 43.1% EPA and 7% DHA, respectively (Kojima, Sakuradani, & Shimizu, 2006).

3.1.3. The *n*−6 polyunsaturated fatty acids

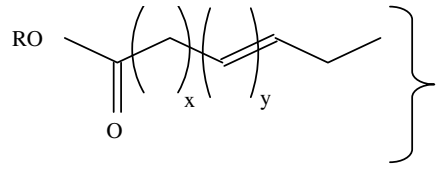
The C_{18}–C_{20} acyl chain length *n*−6 fatty acids like 20:4 *n*−6 and 18:2 *n*−6 exhibited a reduction in their content in triglyceride fraction up to 6 h of lipase-catalysed hydrolysis. The content of arachidonic acid (20:4 *n*−6) was found to be reduced after enzymatic hydrolysis (0.06% TFA after 3 h and 0.05% TFA after 6 h of lipase-catalysed hydrolysis from an initial value of 0.10% TFA) (Table 1). Similarly, among other *n*−6 fatty acids, 18:2 *n*−6 exhibited a reduction of 32.39% after 3 h and 54.93% after 6 h of lipase-catalysed hydrolysis (Fig. 1B). The results also suggest that among *n*−3 and *n*−6 fatty acids, the former is more resistant towards hydrolysis by lipase from *P. fluorescens* MTCC 2421, and the discrimination was the greatest for the C_{18}–C_{20} acyl chain lengthened homologues (EPA and LA). However, after prolonged hydrolysis (9 h), the content of *n*−6 fatty acids recorded a slight increase (0.61% TFA), presumably due to the reduced selectivity of lipase. These results indicate that when there are only a few target fatty acid ester bonds that are susceptible to hydrolysis by a lipase, the lipase can even cleave bonds that are resistant (or nearly resistant) to hydrolysis.

There are earlier reports to enrich γ-linolenic acid (GLA, 18:3 *n*−6) from borage oil by selective esterification with *C. rugosa* lipase. Palmitic, stearic, oleic, and linolenic acids were preferentially esterified compared to 18:3 *n*−6, thus allowing a γ-linolenic acid enrichment (Schmitt-Rozieres, Vanot, Deyris, & Comeau, 1999). An enzymatic method involving seed oil hydrolysis and selective esterification of free fatty acids has been essayed for screening of lipases in order to concentrate 18:3 *n*−6 by using the seed oil of *B. officinalis* and *Echium fastuosum* (López-Martínez, Campra-Madrid, Ramírez-Fajardo, Esteban-Cerdán, & Guil-Guerrero, 2006).

3.2. Structure–bioactivity relationship analyses vis-à-vis fatty acid specificity of lipase towards *n*−3 C_{18}–C_{20} acyl chain length fatty acids

Structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic effect, exemplified by partition coefficient log*P* and bulk parameters MV and *P* to dictate lipase-catalysed hydrolysis of fatty acyl ester linkage of triglycerides. The presence of *n*−3 double bond as in 18:3 *n*−3 and 20:5 *n*−3 reduces the terminal aliphatic chain length (3.7041 Å) that presumably hinders the favourable rearrangement of the active basic aminoacyl residues in the active site, thereby retaining the native form of triglycerides containing *n*−3 fatty acids (Fig. 2A). On the other hand, the extended terminal aliphatic moiety (6.1025 Å) in *n*−6 fatty acids like 18:2 *n*−6 and 20:4 *n*−6 results in favourable rearrangement of the basic aminoacyl residues in the enzyme active site, leading to facile hydrolysis of the ester bond of triglycerides containing *n*−6 fatty acids (Fig. 2B). Irrespective of the presence of *n*−3/*n*−6 moieties, an increase in acyl chain length is directly proportional to degree of hydrolysis. It is apparent that the content of C_{22} fatty acids (C_{22}:*xn*−3, *x* = 5 and 6) with an acyl chain length 25.3461 Å exhibited a reduction of 23.27% and 34.96% in the triglyceride fraction after 3 and 6 h of lipase-catalysed hydrolysis, respectively. The structure–bioactivity relationship analyses of lipases from *Penicillium cyclopium, C. cylindracea, M. miehei, Rhizopus arrhizus*, and *Penicillium* sp. were reported in an earlier study (Mukherjee, Kiewitt, & Hills, 1993). The authors reported that fatty acids having the first double bond from the carboxyl end as a *cis*-4 (22:6 *n*−3), *cis*-6 (18:1 *n*−12, 18:3 *n*−6, 18:4 *n*−3) or a *cis*-8 (20:3 *n*−6) double bond are strongly discriminated, as compared to other fatty acids (Mukherjee et al., 1993). An extracellular alkaline metallolipase with molecular weight of 74.8 kDa derived from cultures of *B. licheniformis* MTCC 6824 was found to enrich Δ5 olefinic double bond fatty acids, viz., EPA and AA (Chakraborty & Paulraj, 2008b).

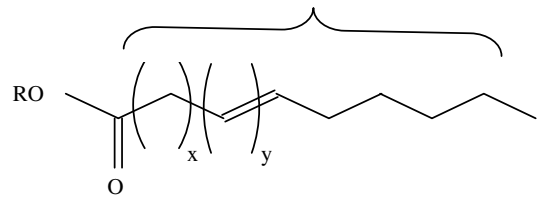
In the present study, the structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic parameter (log*P*) dictating the hydrolytic activity of lipase. The lower hydrophobic constants (log*P* values) of 18:3 *n*−3 and 18:4 *n*−3 (5.65 and 5.33), as compared to that of 18:2 *n*−6 (5.97) apparently results in their higher hydrolytic resistance towards the lipase. Similarly, 20:5 *n*−3 recorded a lower log*P* value (5.85), as compared to its *n*−6 counterpart fatty acid (20:4 *n*−6), which having a higher log*P* value (6.16) is readily attacked by the lipase. Among C_{18} fatty acids, the *n*−3 fatty acids like 18:3 *n*−3 and 18:4 *n*−3 exhibited higher resistance to lipase-catalysed hydrolysis apparently due to the lower value of steric constants like MV and *P* (Fig. 3). For 18:4 *n*−3, the MV and *P* were recorded to be 294.7 and 718.9 cm^3 which



C_{18}-C_{20} *n*-3 fatty acids

***n*-3 Fatty acids**	**x**	**y**
18:3 *n*-3	4	3
18:4 *n*-3	3	4
20:5 *n*-3	2	5

Higher steric parameters (*MV* and *P*) leading to greater lipase-substrate interaction



C_{18}-C_{20} *n*-6 fatty acids

***n*-6 Fatty acids**	**x**	**y**
18:2 *n-6*	4	2
20:4 *n-6*	2	4

Fig. 3. Sketch model of *n*−3 and *n*−6 PUFAs with C_{18}–C_{20} fatty acyl chain length.

were found to be lower than those recorded for 18:2 $n-6$ (MV = 307 cm^3 and P = 744 cm^3). These lower steric parameters in 18:4 $n-3$ presumably lead to greater resistance to hydrolysis by lipase, and increase in the same fatty acid in the triglyceride fraction. The fatty acid 18:3 $n-3$ is comparatively more susceptible to lipase-catalysed hydrolysis than 18:4 $n-3$ apparently due to slightly higher MV (301.1 cm^3) and P (731.7 cm^3) than the latter. However, these values in 18:3 $n-3$ were higher than its $n-6$ counterpart (18:2 $n-6$) resulting in its increased concentration in the triglyceride fraction after 6 h of hydrolysis (8.25 ± 0.61% TFA, from an initial value of 4.47 ± 0.84% TFA). The same hypothesis can be applied to C_{20} fatty acid homologues, to explain the higher rate of hydrolysis of 20:4 $n-6$ than that of 20:5 $n-3$. Higher MV (327.7 cm^3) and P (798.4 cm^3) in 20:4 $n-6$ than 20:5 $n-3$ (MV = 321.3 cm^3 and P = 785.6 cm^3) apparently led to higher hydrolytic resistance of the latter, compared to the former (Fig. 3). This, in turn, leads to an enrichment of 20:5 $n-3$ in the triglyceride fraction after lipase-catalysed hydrolysis. The proportional relationship between the steric parameters and higher hydrolytic susceptibility of the triglycerides, is attributed to the fact that due to an increased surface area, the enzyme exhibits higher hydrolytic activity than the fatty acid triglycerides, having lower surface area with a lower macromolecular receptor interaction at the lipase active site. The steric variables MV and P appeared to influence the bioactivity in a significant manner, and were found to be the most dominant descriptors, which could guide us to devise a biochemical mechanism of lipase activity.

3.3. Urea fractionation of PUFAs

The percent fatty acid composition obtained after urea complexation is illustrated in Table 2. Urea complexation of fatty acids is used to concentrate PUFAs from SFAs and MUFAs, where urea occludes straight-chain compounds such as SFAs and MUFAs in a hexagonal crystalline structure, excluding methylene-interrupted polyunsaturated fatty acids (having –C=C–C–C=C– moiety), due to the irregularities in their molecules caused by the bends at each double bond (Chakraborty & Paulraj, 2007). The interfering SFAs and most of the MUFAs (long- and straight-chain molecules) were removed in the form of urea inclusion complex, while the PUFAs remained in solution. Urea fractionation resulted in dramatic reduction in SFAs (0.49% TFA) and MUFAs (19.53% TFA). Among MUFAs, 18:1 $n-9$ exhibited a marginal reduction of 15.70%, whereas 16:1 $n-7$ was found to be decreased by 55.54% after urea fractionation. The total PUFA after urea fractionation was recorded to be 70.25%, mainly comprising by EPA (42.50 ± 7.15% TFA), 18:3 $n-3$ (10.31 ± 0.60% TFA), and 22:6 $n-3$ (8.22 ± 1.16% TFA). Both 18:4 $n-3$ and 22:6 $n-3$ contents were found to increase 1.54-fold after urea complexation ($p < 0.05$). The urea complexation reaction raised the EPA and LA content by 1.20-fold and 1.25-fold ($p < 0.05$), respectively, thus allowing further purification by argentation column chromatography.

3.4. Separation of LC-PUFAs by argentation column chromatography

Normal-phase vacuum column chromatography over argentated neutral alumina was used to separate different fatty acids according to the differences in their degree of unsaturation (Chakraborty & Paulraj, 2007). The strength of Ag–fatty acid olefinic bond complexation controls the mobility of different fatty acids with variable unsaturation. It is apparent that the preceding urea process removes nearly all SFA and most of the MUFA. Elution of fatty acids with n-hexane/diethyl ether (9.5:0.5, v/v) substantially eliminated the remainder of MUFAs (98.22% TFA) from the urea concentrate. The fractions eluted with n-hexane/diethyl ether 7.5:2.5, v/v followed by n-hexane/CH_2Cl_2 (80:20, v/v) furnished linolenic acid methyl ester of high purity (75.31 ± 5.04% TFA). Docosapentaenoate (22:5 $n-3$, 5.40 ± 0.31% TFA) and docosahexaenoate (22:6 $n-3$, 9.72 ± 0.46% TFA) esters having additional double bonds

Table 2
Percentage composition of individual fatty acids in different solvent eluates by argentation chromatography using urea–fatty acid concentrate

Fatty acids	UA–fatty acid	Eluants (solvent systems)		
		Hexane/diethylether (19:1, v/v)	Hexane/DCM (4:1, v/v)	Hexane/EtOAc (2:1, v/v)
Saturated fatty acids				
12:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
14:0	0.46 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
16:0	0.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
17:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
ΣSFA	0.49	0.00	0.00	0.00
Monounsaturated fatty acids				
16:1 $n-7$	8.15 ± 0.83	28.04 ± 2.17	0.06 ± 0.02	0.00 ± 0.00
18:1 $n-9$	11.38 ± 1.05	70.18 ± 4.35	2.15 ± 0.16	0.12 ± 0.05
17:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20:1 $n-11$	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
ΣMUFA	19.53	98.22	2.21	0.12
Polyunsaturated fatty acids				
18:2 $n-6$	1.57 ± 0.21	0.26 ± 0.03	7.19 ± 0.48	0.18 ± 0.03
18:3 $n-3$	10.31 ± 0.60	0.11 ± 0.02	75.31 ± 5.04	0.27 ± 0.06
18:4 $n-3$	5.33 ± 0.49	0.05 ± 0.01	13.84 ± 0.93	0.19 ± 0.05
20:4 $n-6$	0.83 ± 0.18	0.00 ± 0.00	0.15 ± 0.03	0.14 ± 0.02
20:5 $n-3$	42.50 ± 7.15	0.00 ± 0.00	0.00 ± 0.00	83.62 ± 1.26
22:5 $n-3$	1.49 ± 0.23	0.00 ± 0.00	0.00 ± 0.00	5.40 ± 0.31
22:6 $n-3$	8.22 ± 1.16	0.00 ± 0.00	0.00 ± 0.00	9.72 ± 0.46
ΣPUFA	70.25	0.42	96.49	99.52
EPA/LA	4.12	0.00	0.00	309.70
$n-3$ PUFA	67.85	0.16	89.15	99.20
$n-6$ PUFA	2.40	0.26	7.34	0.32
$n-3/n-6$	28.27	0.62	12.15	310.00
LSD (p = 0.05)	1.98	2.16	2.39	2.85

The notations are as for Table 1. On the basis of the significance of treatments LSD at the 5% level of significance (p = 0.05) was computed. Data presented as mean values of three samples (mean ± standard deviation).

and greater acyl chain length were found to co-elute with EPA, in the fraction containing *n*-hexane/ethyl acetate (2:1, v/v) as the eluting solvent system (Table 2). The fraction eluted with *n*-hexane/diethyl ether (3:2, v/v) followed by *n*-hexane/EtOAc (2:1, v/v) yielded EPA methyl ester in high purity (83.62 ± 1.26% TFA) (Table 2).

4. Conclusions

In conclusion, the present study showed the change in different classes of fatty acid composition as a function of time course (3–9 h) hydrolysis of triglycerides from sardine oil by a lipase purified from *P. fluorescens* MTCC 2421. The C_{18}–C_{20} acyl chain lengthened $n-3$ PUFAs (linolenic acid and eicosapentaenoic acid) in the triglyceride mixture increased proportionally (1.98-fold increase) with the progress of hydrolysis up to 6 h; after that their concentration plateaued, due to reduced selectivity of the lipase. It was found that ester bonds of C_{18}–C_{20} acyl chain lengthened fatty acid homologues are highly resistant towards hydrolysis by lipase from *P. fluorescens* MTCC 2421, resulting in their enrichment in fatty acid triglycerides. Structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic effect, exemplified by partition coefficient log*P* and bulk parameters molecular volume and parachor, to dictate lipase-catalysed hydrolysis of fatty acyl ester bonds of triglycerides. The urea complexation reaction raised the eicosapentaenoic acid and linolenic acid content by 1.20-fold and 1.25-fold, respectively, thus allowing further purification by argentation column chromatography. The fractions eluted with *n*-hexane/CH_2Cl_2 (80:20, v/v) and *n*-hexane/EtOAc (2:1, v/v) by argentation chromatography furnished linolenic acid and eicosapentaenoic acid methyl ester to a final purity of 75.31 ± 5.04% and 83.62 ± 1.26% TFA, respectively. It can be concluded that a combination of *P. fluorescens* MTCC 2421 lipase-catalysed hydrolysis followed by urea fractionation and argentation chromatography is a promising method to separate and concentrate C_{18}–C_{20} PUFAs with $n-3$ double bonds, like eicosapentaenoic acid and linolenic acid.

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