Preparation of eicosapentanoenic acid concentrates from sardine oil by *Bacillus circulans* lipase

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**A R T I C L E  I N F O**

Article history:
Recieved 27 May 2009
Received in revised form 1 September 2009
Accepted 13 October 2009

Keywords:
Lipase
*Bacillus circulans*
Sardine oil
\(n\)-3 PUFA
Amide complexation
Argentated chromatography

**A B S T R A C T**

An extracellular lipase derived from *Bacillus circulans*, isolated from marine macroalga, *Turbinaria conoides*, was used to prepare \(n\)-3 polyunsaturated fatty acid (PUFA) concentrates from sardine oil triglycerides. The enzyme was purified 132-fold with specific activity of 386 LU/mg. The purified lipase was able to enrich sardine oil with 37.7 ± 1.98% 20:5\(n\)-3 and 5.11 ± 0.14% 18:3\(n\)-3 in the triglyceride fraction after 3 h of hydrolysis. Lower hydrophobic constants of \(n\)-3 fatty acids (18:3\(n\)-3 log \(P_{18:3}\) = 5.65; 20:5\(n\)-3 log \(P_{18:5}\) = 5.85, respectively) than \(n\)-6 (20:4\(n\)-6 log \(P_{20:4}\) = 6.16) resulted in higher hydrolytic resistance of the former toward lipase, leading to their enrichment in the triglyceride fraction. Lipase-catalysed hydrolysis of sardine oil for 3 h, followed by urea complexation, provided free fatty acids containing 51.3 ± 4.65% 20:5\(n\)-3. The purified methyl ester of 20:5\(n\)-3 (68.29 ± 2.15%) from the urea concentrate was attained by chromatography on argentated neutral alumina.

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1. Introduction

The long chain \(n\)-3 polyunsaturated fatty acids (PUFAs) with \(C_{20}\) acyl chain length, particularly eicosapentanoenic acid (EPA, 20:5\(n\)-3) and linolenic acid (LA, 18:3\(n\)-3), were reported to have various important physiological roles in health, and in the inflammatory processes (Sahena et al., 2009). Whilst saturated and monounsaturated fatty acids may be synthesised in the body, PUFAs cannot be synthesised \textit{de novo} due to the lack of essential enzymes required to synthesise PUFA in adequate levels from precursor fatty acids, and therefore, must be externally supplied in the diet (Cahu, Guil-Guerrero, Rincon-Cervera, & Guil-Guerrero, 2005). Also fishery by-products/processing waste and low value catches have been utilised for concentrating PUFA (Zuta, Simpson, Chan, & Phillips, 2003). A survey of the literature revealed a diverse range of methods for enrichment and purification of LC-PUFAs. These included urea inclusion complexation, molecular distillation, iodolactonisation, low temperature fractional crystallisation, salt solubility methods, and liquid–liquid extraction–fractionation using aqueous AgNO\(_3\) solution (Guil-Guerrero et al., 2007).

However, most of the existing chemical methods for purifying individual PUFAs and PUFA concentrates are non-selective for different fatty acids. The substrate specificity of lipases (triacylglycerol acyl hydrolases) has been utilised for the enhancement of PUFA content in fish oils by several groups (Can & Özçelik, 2005; Liu, Zhang, Hong, & Ji, 2007). It was found that lipases were efficient in hydrolytic and esterification reactions because of their high stereospecificity and mild reaction conditions at low temperature and in organic solvents (Can & Özçelik, 2005). The unique specificity of lipases is utilised to selectively concentrate targeted fatty acids in the triacylglyceride fraction that can be readily absorbed into plasma triacylglycerol.

Bacterial lipases are one of the predominant classes of lipases for the enhancement of PUFA content in fish oils, due to their unique thermostability. Lipases have been reported from *Bacillus coagulans* (Kumar, Kikon, Upadhyay, Kanwar, & Gupta, 2005), *B. circulans* (Kademi, Ait-Abdelkader, & Fakhreddine, 2000), and *B. licheniformis* (Chakraborty & Paulraj, 2008a), that were used to concentrate \(n\)-3 PUFAs. Novozym 435\textsuperscript{TM} was used to selectively concentrate targeted fatty acids in triglycerides due to its unique substrate specificity towards PUFAs (Liu et al., 2007). Response surface methodology was used to determine optimal conditions for the lipase-catalysed enrichment of hazelnut oil by incorporating \(n\)-3 PUFAs from menhaden oil (Can & Özçelik, 2005). DHA-rich
triglycerides were prepared from fish oil with lipases obtained from *Candida cylindracea* and *Chromobacterium viscosum* (Tanaka, Hirano, & Funada, 1994).

PUFAs are found to be widely available in marine fish species. However, sardine (*Sardinella longiceps*) is a low-value fish, and its oil is locally available as a by product. A higher concentration of PUFAs (31.4%) particularly EPA (17.6%) in sardine oil is an added advantage. In this paper, we report methods to purify an extracellular lipase, to homogeneity, from culture broth of *B. circulans*, originally isolated from marine macroalga *Turbinaria conoides*. The present paper is also directed to a process to prepare n-3 PUFA concentrates by lipase-catalysed purification of triglycerides derived from sardine oil, and further concentrating the target PUFAs by urea complexation and argentated column chromatographic methods. Structure–activity relationship analyses, to correlate the lipase activity with physicochemical properties of different classes of fatty acids, were used to elucidate the structural descriptors of fatty acids (hydrophobic and steric) controlling lipolytic activity.

2. Materials and methods

2.1. Chemicals and instrumentation

The solvents used for sample preparation were of analytical grade (E-Merck, Darmstadt, Germany). Sephadex G-100 and other supports of chromatography were from Bioscience Research Laboratories (SRL, Mumbai, India). Amberlite IRA-410 (Cl− form) and bovine serum albumin were obtained from HiMedia (Mumbai, India); Electrophoresis grade acrylamide, bis-acrylamide, and low-range molecular marker proteins were procured from Bio-Rad Laboratories (Bio-Rad, Hercules, CA). All glassware was rinsed with CHCl3/CH3OH (2:1, v/v), and dried under N2. Standards of fatty acid methyl ester (Supelco TM 37 Component FAME Mix, Catalog No. 47885-U) were used to elucidate the structural descriptors of fatty acids, were used to elucidate the structural descriptors of fatty acids (hydrophobic and steric) controlling lipolytic activity.

2.2. Isolation of *B. circulans* from *T. conoides* and lipase production

For isolation of epiphytic bacteria, marine macroalga *T. conoides* samples obtained from the Gulf of Mannar were rinsed with sterile seawater, and a small portion of the algal surface was scraped with a sterile swab and spread onto the plates of marine agar plates. After incubation at 37 °C for 24-48 h, all colonies were streaked onto the marine agar slants and maintained for further microbiological and biochemical identification. Standard sampling procedures were followed, and colonies were recovered from marine agar plates (Chakraborty & Paulraj, 2008a). The bacterial cultures obtained from the alga were thereafter grown on modified tributyrin slants (0.3% meat extract, 0.5% peptone, 1% NaCl, 0.3% tributyrin, and 0.01% CaCl2·2H2O) to detect the lipolytic activity. Amongst several epiphytic bacteria isolated from this alga, one *Bacillus* sp. (*B. circulans*) was used for production of lipase due to its higher lipolytic potential. The bacterial cultures belonging to *B. circulans* were inoculated in a 250 ml Erlenmeyer flask containing nutrient broth (100 ml). The content was then incubated at 37 °C for 24 h under shaking (150 rpm) to raise the inoculum for the enzyme production. The seed culture (100 ml) (1% v/v) of *B. circulans* was grown in sterilised broth, containing (g l−1): NaNO3 3; K2HPO4 0.1; MgSO4·7H2O 0.5; KCl 0.5, FeSO4·7H2O 0.05, yeast extract 3.0, and microalgae paste (*Isochrysis galabana*, 5%, v/v) at 37 °C in a 1000 ml Erlenmeyer flask with shaking (150 rpm) up to 72 h. The culture broth was harvested at different time-intervals (0, 5, 10, 24, 29, 34, 48, 53, 58, and 72 h) to determine the optimum time for maximum lipase production. The culture broth was clarified by centrifugation (10,000g for 20 min at 4 °C, Superspin R-V/ FM Plasto Crafts, Plasto Crafts Mumbai) to recover the supernatant that was filtered (0.2 μ). This concentrated liquid, referred to as the crude extracellular lipase solution, was used for further purification.

2.3. Purification of lipase from *B. circulans* by ammonium sulphate precipitation and chromatography

The crude lipase thus obtained was purified to homogeneity by ammonium sulphate precipitation, Sephadex G-100 gel filtration, and Amberlite IRA-410 (Cl− form) anion-exchange chromatography. Solid ammonium sulphate was added to the crude enzyme extract in increments of 5% until 70% saturation (w/v) with gentle stirring for 2 h at 4 °C, and allowed to stand for 4 h. The precipitates thus obtained were separated by centrifugation (10,000g for 30 min, 4 °C), and the supernatant was fractionated with the slow addition of ammonium sulphate to 70% saturation. The solution was stirred for 1 h at 4 °C, and allowed to stand overnight. The precipitated proteins were collected by centrifugation at 10,000g (30 min, 4 °C), and the supernatant was discarded. The pellet was dissolved in a minimum volume of Tris–HCl buffer (50 mM, pH 8.0), and dialysed against the same buffer for 18 h to remove the residual salts, and assayed for lipase activity (Winkler & Stickmann, 1979). The enzyme concentrate obtained from dialysis was loaded onto a Sephadex G-100 gel filtration column (2.5 × 120 cm) at a flow rate of 0.5 ml/min. The column was equilibrated and eluted with Tris–HCl buffer (50 mM, pH 8.0) supplemented with CaCl2 (1.0 mM). The eluant fractions showing lipase activity (obtained at the 22–30th fraction) were pooled together, and concentrated by lyophilisation. The lyophilised protein was dissolved in distilled water (5 ml), and dialysed against Tris–HCl buffer (50 mM, pH 8.0). The concentrated protein was further chromatographed on an Amberlite IRA-410 (Cl− form) column (1.5 × 15 cm) pre-equilibrated with Tris–HCl buffer (50 mM, pH 8.0), and the bound proteins were eluted in Tris–HCl (50 mM, pH 8.0) with a linear gradient of increasing concentration of NaCl (0–0.5 M) at a flow rate of 0.5 ml/min. The active fractions (obtained at the 35–40th fraction) were pooled, and analysed for lipase activity. The lipase activity of the enzyme was estimated spectrophotometrically (Varian Cary 50 Conc., USA), using 4-nitrophenyl palmitate (4-NPP) as reported earlier (Winkler & Stickmann, 1979). One activity unit of lipase (LU) was defined as μmole of 4-NP released from hydrolysis of 4-NPP/ml/min by one ml of enzyme under standard assay conditions. The protein concentration was determined by measuring the absorbance at 590 nm (Varian Cary 50 Conc., USA) using bovine serum albumin (20–150 μg) as a standard (Bradford, 1976). The active enzyme fractions were stored at 4 °C until used for polyacrylamide gel electrophoresis and further enzyme characterisation.

2.4. Determination of molecular weight of the lipase by denaturing polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (0.1% v/v SDS–PAGE) was performed on 12% polyacrylamide gel (with 6% stacking gel), following the established procedure (Laemmli, 1970), and the apparent molecular mass of proteins was determined with reference to the low-range molecular mass markers (14.4–97.4 kDa, Bio-Rad Laboratories, Hercules CA). The molecular mass markers used were *b*-phosphorylase (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor
(21.5 kDa) and lysozyme (14.4 kDa). After migration, the gels were fixed using 7% (v/v) CH₃COOH/CH₃OH, and submitted to a cycle of staining/destaining with Coomassie Brilliant Blue R-250 (stainer) and 14% (v/v) CH₃COOH/CH₃OH (destainer), respectively. Molecular mass was determined from the plots of log molecular mass (log M) vs. migration (R₀) for the series of protein standards. The active lipase fractions were identified by analytical SDS–PAGE of proteins, using 12% gels stained with Coomassie brilliant blue R-250 as detailed elsewhere (Kim, Kwon, Yoon, Kim, & Kim, 2005). The gels were rinsed with water and stored in acetic acid (7% v/v) (Lee, Chung, & Rhee, 1993) to develop a dark yellow colour. Gel filtration chromatography with Sephadex G-75 was used to validate the molecular weight of lipase. The elution volume of the lipase was being the elution volume of blue dextran (2000 kDa).

2.5. Preparation of n-3 polyunsaturated fatty acid concentrates by time-course (1–6 h) B. circulans lipase-catalysed hydrolysis of sardine oil triglycerides

In this study, crude sardine oil from whole sardine was obtained from a plant located in Cochin, India. The crude oil thus obtained was chilled and filtered to remove solid impurities. The content was further bleached by the addition of powdered activated charcoal (4%) to the oil under vacuum conditions, to remove coloured compounds and other impurities (metals such as Ni and Fe) from the oil. The powder was then removed by filtration, and the product was treated for a short time at high temperature to remove low molecular weight contaminants (free fatty acids, volatile amines) and peroxides. Antioxidant (tertiary butyl hydroxyquinone 0.01% w/v) was added to the oil to arrest oxidation. The free flowing light yellow-coloured oil thus obtained was stored under nitrogen at −20 °C, in a sealed dark amber glass container, until used.

Sardine oil triglycerides thus obtained were further stabilised by adding phosphatidylcholine and α-tocopherol (0.1% each) and metal complexing agents (ascorbic acid, 0.01%). The triglycerides from sardine oil were cleaned up on neutral alumina, using n-hexane/ETOEt as the eluting solvent system (95:5, v/v), and extracted, following established method (Bligh & Dyer, 1959). The lipids thus obtained were further purified on a column of neutral alumina, using n-hexane/ETOEt (95/5, v/v), and concentrated under reduced pressure to remove free fatty acids (Chakraborty & Paulraj, 2008b). The upper n-hexane layer was concentrated at 40 °C in vacuo, and the concentrated glycerides were maintained under N₂ at −20 °C prior to further use. The hydrolysis products of the reaction were monitored by thin-layer chromatography, using silica gel as adsorbent and CHCl₃/Me₂O/AcOH (80:20:0.5, v/v/v) as developing solvent system, and visualised by exposure to iodine vapour. To obtain pure triacylglycerol from the n-hexane layer, the triacylglycerols were separated on an alumina column as eluted with n-hexane/ETOEt (9:1, v/v). Free fatty acids from triacylglycerol, obtained after saponification (Metcalf, Schmizt, & Pleka, 1966), were derivatised to their methyl esters and N-acyl pyridolides, following established procedure (Andersson, 1978) for gas–liquid chromatographic (GLC) and gas chromatographic–mass spectroscopic (GC–MS) analyses (Chakraborty & Paulraj, 2008b). Various physico-chemical descriptors, namely partition coefficient (log P) and bulk parameters (molar volume MV and pararoch P) (Chakraborty & Devakumar, 2005; Chakraborty, Devakumar, Tomar, & Kumar, 2003) have been used to understand the structural features of the fatty acids dictating lipidic activity.

2.6. Urea complexation and argentated column chromatographic fractionation of individual PUFAs

The PUFAs were concentrated by urea-fatty acid complexation, as detailed elsewhere (Guill-Guerrero et al., 2007). Briefly, the triglycerides were saponified to furnish free fatty acids (3 g), to which urea (12 g) in aqueous MeOH (120 ml) was added, and the contents were heated (60–65 °C) until a clear homogeneous solution was obtained (Chakraborty & Paulraj, 2007). 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 HCl (5 ml, 1%, v/v). The mixture was further extracted with n-hexane (20 ml × 2), and the combined organic layers were washed with water, and dried over anhydrous Na₂SO₄, before being evaporated. The resulting PUFA concentrate obtained by urea complexation was dissolved in MeOH, and transesterified to furnish the FAMEs, which were extracted with n-hexane, and concentrated under reduced pressure to yield a residue. The residue was dissolved in n-hexane (30 ml) to be applied onto the argentation chromatography column, and for analysis by GC/GC–MS. An aliquot of crude methylated extract (25 mg) obtained in the transesterification step was dissolved in n-hexane (5 ml) and subjected to liquid chromatography over argentated neutral alumina as detailed elsewhere (Chakraborty & Paulraj, 2007), and the cold-water jacketed column was eluted with n-hexane/Me₂O and a step gradient, increasing the proportion of Me₂O (99:1 to 90:10, v/v) to provide ten fractions (Fr-F₁₀, 30 ml each). Hexane was used to remove the non-polar fraction, followed by Me₂O to give the polar fraction. The fraction eluted by n-hexane/Me₂O (9:1, v/v) was further separated using a stepwise gradient system (n-hexane to EtOAc). Evaporation of solvents from fractions, followed by silver-ion thin-layer chromatography (AgNO₃/TLC, 5 cm × 20 cm) using n-hexane/ETOEt/AcOH (80:20:0.5, v/v/v), validated the purity of the individual fatty acids. The TLC bands were stained with 2,7-dichlorofluorescein in MeOH (0.1%, w/v), and examined under UV-light.

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

The fatty acid composition of the PUFA concentrate was determined as described elsewhere (Chakraborty & Paulraj, 2007). Briefly, the triglycerides were extracted by using CHCl₃/MeOH/H₂O (2:4:1, v/v/v), and saponified with alkaline reagent (0.5 N KOH/MeOH). The saponifiable materials were extracted with
petroleum ether:EtOEt (1:1, v/v) after removal of nonsaponifiable materials by solvent extraction (with n-hexane) and acidification (1 N HCl). A methylation mixture (14% BF₃/CH₃OH) was used to transesterify the saponifiable material, yielding fatty acid methyl esters (FAME) that were extracted with n-hexane/H₂O. 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 AutoSystem XL, Gas chromatograph (Perkin-Elmer, USA) equipped with a flame ionisation detector (FID) analysed the composition of the 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, Bellfonte, PA). The oven temperature was set 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. He 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). The El/EC–MS analyses were performed on a single-quadrupole mass spectrometer (Varian 1200L, USA) under electron impact (EI, ionisation energy 70 eV) conditions, with an on-column injector set at 110 °C for confirmation of the fatty acids identification. FAMES were derivatised to N-acetyl pyrrolidines 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 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 non-polar (VF–5MS, 5% phenyl substituted methylsiloxane). The carrier gas was of ultra high 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 analysed using Varian Workstation (version 6.2) software. The recovered fatty acid methyl esters (FAMES) from urea fractionation were resolved by TLC (5 cm × 20 cm), pre-coated with silica gel and impregnated with AgNO₃ following a reported method (Chakraborty & Paulraj, 2007). TLC plates were developed twice in n-hexane/diethyl ether/acetic acid (80:20:0.5, v/v/v) to separate individual bands. The bands were stained with 2,7-dichlorofluorescein in alcohol (0.1%, w/v), and examined under UV-light.

2.8. 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 using SPSS (version 10.0) 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.

3. Results and discussion

3.1. Preparation of crude lipase from B. circulans

Lipase-selective medium (rhodamine B-triolein agar) was used to determine the lipase-producing capability of B. circulans isolated from the marine macroalga T. conoides. The crude extracts obtained after 24 h of incubation exhibited a lipase activity of 7.3 ± 0.8 LU/ml. However, the crude extract obtained after 34 h of incubation recorded the highest lipase activity (12.2 ± 1.1 LU/ml) (Fig. 1A), and was therefore used for further purification of the lipase. Earlier studies indicated maximum lipase activities at the preset of logarithmic phase of bacterial growth (Schuepp, Kermasha, Michalski, & Morin, 1997).

3.2. Purification of B. circulans lipase and molecular studies

The results of purification profile of the extracellular lipase by B. circulans are summarised in Table 1. A 109-fold purification with a specific activity of 119 LU/mg was attained after gel filtration on Sephadex G-100 (Table 1). Highest lipase activity was apparent at the 26th fraction of the gel filtration (Fig. 1B). The enzyme was finally purified 132-fold, using Amberlite XRD-5 (Cl−form) anion-exchange chromatography, at the 32nd fraction, with a specific activity of 386 LU/mg (Fig. 1C and Table 1). The homogeneity of the purified lipase was checked by the presence of a single band corresponding to an apparent molecular mass of 39.8 kDa on SDS–PAGE gels, suggesting it to be a homomeric protein (Fig. 1D). Activity staining confirmed the presence of the purified lipase. Gel filtration chromatography, using Sephadex G-75, indicated the apparent molecular weight of the lipase as 40.5 kDa.

3.3. Change in fatty acid composition as a function of time-course (1–6 h) lipase-catalysed hydrolysis of triglycerides from sardine oil

3.3.1. General

The refined sardine oil was found to contain PUFAs, particularly 18:3n-3 (2.5 ± 0.5%), 20:5n-3 (17.6 ± 0.6%) and 22:6n-3 (8.2 ± 0.9%), along with other n-3 and n-6 PUFAs, as indicated in Table 2. The n-3 fatty acids contributed the major share (30.6%) with the n-3/n-6 fatty acid ratio of 13.3. Amongst saturated fatty acids, 16:0 was found to be predominant, followed by 14:0, and 18:0 (Table 2). The sardine oil triglycerides were hydrolysed with lipases purified from B. circulans; the total fatty acid content of triglycerides at various time-intervals of hydrolysis (1–6 h) were analysed, and the results are indicated below.

3.3.2. Saturated (SFAs) and mono-unsaturated fatty acids (MUFAs)

The fatty acid composition of sardine oil and those of enzyme hydrolysates are given in Table 2. The lipase produced the highest degree of hydrolysis for SFAs (47.2%), followed by MUFAs (25.9%), from their initial content after 3 h. The total concentration of SFA exhibited reductions of 15.1% and 52.2% after 1 and 3 h of hydrolysis, respectively whereas, after prolonged hydrolysis (6 h), the proportion of SFAs to total fatty acids was found to be increased (18.5%) (Table 2). The total MUFA content was found to be reduced by 4.8% after 1 h of lipase-catalysed hydrolysis, and 21.9% after 3 h. However, the proportion of total MUFAs exhibited an increase (25.5%), even after 6 h of hydrolysis (Table 2). The decrease in the contents 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 polyunsaturated fatty acids, resulting in their enrichment in the triglyceride fraction (Fig. 2A). It was reported that those substrates containing D2–D7 isomers of 18:1 were resistant to lipase-catalysed hydrolysis, resulting in higher concentration of the fatty acid, and the discrimination was greatest for the D7 isomer (Heimermann, Holman, Gordon, Kowalyshyn, & Jensen, 1973). 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 lower content of these fatty acids (Chakraborty & Paulraj, 2008b).
3.3.3. The n-3 polyunsaturated fatty acids (n-3 PUFAs)

The percentages of n-3 PUFAs released by lipase as a function of time is illustrated in Fig. 2B. The lipase was able to enrich sardine oil with 37.7 ± 1.9% of 20:5n-3 after 3 h of hydrolysis of triglycerides, which was higher than that in the crude sardine oil (Table 2). The total n-3 PUFAs increased with time up to 3 h of lipase-catalysed hydrolysis (52.7%), beyond which it plateaued (51.7% at 6 h), apparently due to the reduced selectivity of the lipase. Other n-3 PUFAs with a C18 acyl side chain, e.g. 18:3n-3 and 18:4n-3, also exhibited an increasing trend in their concentration after 1 and 3 h of lipase-catalysed hydrolysis (Table 2). After 3 h, these fatty acids were recorded as 5.1 ± 0.1% and 2.3 ± 0.1%, respectively, and significantly higher than the initial content (p = 0.05). However, with increase of acyl chain length (>C20), hydrolytic susceptibility of...
the ester linkage of triglycerides (by the lipase) was found to increase as is evident from the 12.1% decrease of 22:6n-3 in the triacylglyceride fraction after 3 h of hydrolysis. The final 22:6n-3 content in the glyceride mixture after 6 h hydrolysis was 6.5 ± 0.6%, i.e., 1.3% less than in the original sardine oil (Table 2). Similar results have been apparent from the reduced content of 22:5n-3 (Table 2).

Generally lipases are very specific in hydrolysing specific fatty acids with specific acyl chain length (e.g. C20 or C22 acyl chain length) or specific classes of fatty acids (e.g. EPA with C20 acyl chain length and DHA C22 acyl chain length). Since lipase reaction is very specific, some lipases (e.g. the B. circulans lipase in this study) can concentrate a particular fatty acid (e.g. EPA, 20:5n-3), but may be ineffective for concentrating other fatty acids (e.g. DHA 22:6n-3, which could not be enriched in this process). The aim of this study is to enrich EPA (20:5n-3) from sardine oil. The bacterial lipase purified from B. circulans is specific in hydrolysing fatty acids higher than C20 n-3, e.g. DHA, thereby decreasing their concentration in the triglyceride fraction. Earlier studies indicated the increase in n-3 fatty acids (63.7%) in the triacylglycerol after 6 h of hydrolysis by an extracellular lipase isolated from B. licheniformis (Chakraborty & Paulraj, 2008b). A 2-fold increase of DHA and EPA concentration was reported by Pseudomonas cepacia and Candida rugosa lipase-catalysed hydrolysis of Salmo salar L. oil (Sun, Pigott, & Herwig, 2002). A microbial lipase isolated from C. antarctica was reported to yield 93.5% PUFA with 25.7% EPA and 44.7% DHA from cod liver oil triglycerides (Medina et al., 1999). Hydrolysis by P. fluorescens HU380 lipase, followed by amide complexation of cod liver oil, was reported to yield 43.1% EPA and 7% DHA (Kojima, Sakuradani, & Shimizu, 2006). From these results it can be concluded that the bacterial lipase is specific in hydrolysing fatty acids higher than C20 n-3. It is apparent that a particular fatty acid, e.g. 22:5n-3 or 22:6n-3, released on hydrolysis is more abundant, resulting in decrease of the triacylglycerol portion of fatty acids, thus suggesting that the ester bond of the fatty acids are susceptible to hydrolysis by the lipase used in the present study. On the other hand, 20:5n-3 and 18:3n-3 (having the C18 n-3 configuration) are more abundant in the triacylglycerol portion of fatty acids, thus suggesting that the lipase is less reactive towards that fatty acid’s ester bond (resistant to hydrolysis), resulting in their increase in the triglycerides (Chakraborty & Paulraj, 2008b). However, due to its acyl side chain and olefinic double bond specificity, this particular lipase obtained from B. circulans is not able to concentrate DHA.

3.3.4. The n-6 polysaturated fatty acids (n-6 PUFAs)

In general, the n-6 fatty acids exhibited a reduction in their content after lipase-catalysed hydrolysis. Amongst n-6 PUFA’s, 18:2n-6 was recorded to be 0.5 ± 0.1% after 1 h of lipase-catalysed hydrolysis, and 0.4 ± 0.1% after 3 h, which were substantially lower than that in the crude sardine oil (Table 2). Similarly, the content of 20:4n-6 in triglyceride was found to decrease by 94.3% after 1 h of hydrolysis, and 95.6% after 3 h (Table 2). The results apparently suggest that, amongst n-3 and n-6 fatty acids, the former are more resistant to hydrolysis by the lipase used in this study, and the discrimination was the greatest for the C18-20 acyl chain-length homologues. However, after prolonged hydrolysis (9 h), the content of n-6 fatty acids recorded a slight increase, presumably due to the reduced selectivity of lipase. It is apparent that, after prolonged hydrolysis, only a few target fatty acid ester bonds (n-6 fatty acyl ester bonds) are available in the reaction mixture that are susceptible to hydrolysis by a lipase. The microbial lipase can even cleave bonds that are resistant (or nearly resistant) to hydrolysis (C18-20 n-3 fatty acids like EPA) to maintain the dynamic equilibrium of the system. These results indicate that the resistance of hydrolysis of this latter class of fatty acyl ester bond depends upon the presence of other substrates (n-6 fatty acyl ester bonds and esters other than C18-C20 n-3 fatty acids) in the reaction mixture.

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**Table 2**

<p>| Fatty acid composition (as weight %) of crude and lipase hydrolysate of sardine oil at three different time durations (1, 3, and 6 h) using purified lipase obtained from <em>B. circulans</em>, followed by urea complexation. |
|---------------------------------|-----------|-----------|-----------|</p>
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Sardine oil</th>
<th>Fatty acid (as weight %)</th>
<th>UA-fatty acid*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h</td>
<td>3 h</td>
<td>6 h</td>
<td></td>
</tr>
<tr>
<td><strong>Saturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>8.5 ± 0.2</td>
<td>8.3 ± 0.3</td>
<td>5.0 ± 0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>18.1 ± 1.9</td>
<td>17.1 ± 0.2</td>
<td>9.3 ± 0.2</td>
</tr>
<tr>
<td>17:0</td>
<td>13 ± 0.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18:0</td>
<td>21 ± 0.0</td>
<td>25.4</td>
<td>14.3</td>
</tr>
<tr>
<td><strong>Monounsaturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1n-7</td>
<td>16.1 ± 1.8</td>
<td>15.4 ± 1.7</td>
<td>11.2 ± 1.0</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>19.9 ± 2.7</td>
<td>19.0 ± 1.3</td>
<td>17.3 ± 1.6</td>
</tr>
<tr>
<td>20:1n-11</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td><strong>Palmitoleic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1n-9</td>
<td>36.6</td>
<td>34.8</td>
<td>28.7</td>
</tr>
<tr>
<td><strong>Polyunsaturated fatty acids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>0.7 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>2.5 ± 0.5</td>
<td>3.3 ± 0.3</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>18:4n-3</td>
<td>1.2 ± 0.3</td>
<td>1.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>1.6 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>17.6 ± 0.6</td>
<td>23.0 ± 1.3</td>
<td>37.7 ± 1.9</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>1.1 ± 0.2</td>
<td>0.5 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td><strong>SFA</strong></td>
<td>43.8</td>
<td>41.9</td>
<td>37.7</td>
</tr>
<tr>
<td><strong>PUFA</strong></td>
<td>56.2</td>
<td>58.1</td>
<td>62.3</td>
</tr>
<tr>
<td><strong>SFA:total saturated fatty acid</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>PUFA:total polyunsaturated fatty acid</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* UA-fatty acid concentrate implies the urea concentrate of fatty acids. ΣSFA: total saturated fatty acids; ΣMUFA: total monounsaturated fatty acids; ΣPUFA: total polyunsaturated fatty acids; Data presented as mean values of three samples (means ± standard deviation). At the beginning of the hydrolysis (5 h) the lipases display a significant preference for n-3 PUFAs containing 18–20 carbon atoms in the acyl chain. However, the resistance to release of SFAs and MUFAs was less as the hydrolysis reaction progressed. These values do not total 100% because minor fatty acids are not reported. ND implies non-detectable (or fatty acids present below 0.05%).
earlier study reported that the content of 20:4n-6 in the glyceride mixture increased with the progress of hydrolysis by lipase purified from *B. licheniformis* (Chakraborty & Paulraj, 2008b). This was found to be due to fatty acid specificity of lipases toward PUFA:s with Δ5 unsaturated double bond (cis-5 olefinic bond positioned at the number 5 carbon from the carboxyl end) (Chakraborty & Paulraj, 2008b). Earlier results indicated that the lipase from *B. licheniformis* was unique in the resistance it encountered with 20:5n-3 and 20:4n-6. This could be attributed to the positional specificity of the enzyme at the olefinic double bonds of PUFA:s. Lipases have been screened from different sources, to selectively concentrate γ-linolenic acid from *B. officinalis* and *Echium fastuosum* seed oil (López-Martínez et al., 2004).

3.4. Specificity of bacterial lipase towards different fatty acids and structure–bioactivity relationship analyses

A direct hydrophobic effect, as exemplified by partition coefficient log P and bulk parameters molar volume (MV) and parachor (P), appeared to dictate lipase-catalysed hydrolysis of the fatty acyl ester linkage of triglycerides. The n-3 fatty acids exhibited lower hydrophobic activities (20:5n-3, log P = 5.85) than did the n-6 fatty acids (20:4n-6, log P = 6.16), resulting in greater hydrolytic susceptibility of the latter, leading to n-3 decrease in the triglyceride fraction. Similarly, the n-3 fatty acid 18:3n-3 showed higher hydrolytic resistance to lipase, evidently due to the lower bulk (MV = 301 cm³; P = 731 cm³). The fatty acid 22:5n-3 is comparatively more susceptible to lipase-catalysed hydrolysis than is 20:5n-3, apparently due to the slightly higher MV and P than in the latter, thereby increasing the content of 20:5n-3 in the triglyceride fraction. The terminal double bond at the n-3 position (between C₁₅–₁₆ moiety of 18:3n-3 and 20:5n-3) appeared to reduce the aliphatic chain (3.7041 Å) that apparently hinders the favourable interaction of the aminoacyl residues in the active site. This, in turn, appeared to retain the native form of triglycerides containing n-3 fatty acids (Fig. 3). The fatty acid 20:5n-3 has a lower bulk than has 20:4n-6, as exemplified by its lower MV (321 cm³) and P (798 cm³) than the latter. It is apparent that an extended terminal aliphatic moiety (6.1025 Å) in the n-6 fatty acid homologues (18:2n-6 and 20:4n-6) results in favourable rearrangement of the aminoacyl residues in the enzyme active site leading to facile hydrolysis of ester bonds of triglycerides containing n-6 fatty acids (Fig. 3). These results indicated that *B. circulans* lipase is unique in its lack of activity on n-3 PUFA:s. The decrease in the contents of SFAs and MUFA:s in the triglyceride fraction with the progress of hydrolysis suggests that they are more easily hydrolysed by the lipase than are n-3 fatty acids, resulting in enrichment of the latter in the triglyceride fraction. The bacterial lipase from *B. circulans* is specific in hydrolysing fatty acids higher than C₁₉, n-3, thereby concentrating EPA (C₂₀ n-3 fatty acid). The content of C₂₂ fatty acids (22:5n-3) with an acyl chain length of 25.3461 Å exhibited a reduction in the triglyceride fraction after lipase-catalysed hydrolysis. Similarly 22:6n-3 was reduced by 4.0% after 1 h of hydrolysis. However, after prolonged hydrolysis of triglycerides (3 and 6 h), the content of this fatty acid exhibited a reduction in the hydrolysis rate (12.1% and 20.6%, respectively) apparently due to the saturation of lipase activity. Such fatty acid selectivity of lipases, i.e., hydrolysis favouring n-6 over n-3, and long-chain fatty acids (>C₂₀) (22:6n-3) over 20:5n-3, may therefore be useful for fractionating the latter from sardine oil. It is noteworthy that the microbial lipase from *B. circulans* used in the present study was specific in hydrolysing n-6 fatty acids and n-3 fatty acids with >C₂₀ acyl chain length (22:6n-3) at the initial stage of hydrolysis (up to 6 h) of fatty acyl ester bonds. However, after prolonged hydrolysis, when only a few target fatty acyl ester bonds (n-6 fatty acyl ester bonds and esters other than C₁₈–₂₀ 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 to hydrolysis (C₁₈–₂₀ n-3 fatty acids). An earlier study suggested that the fatty acid triacylglycerols containing Δ6 and Δ9 isomers were more susceptible to lipolysis by *B. licheniformis* MTCC 6824 lipase, and the discrimination was the greatest for the Δ5 isomer (20:5n-3 and 20:4n-6), followed by Δ4 double-bonded fatty acids such as 22:6n-3 (Chakraborty & Paulraj, 2008b). Structure–activity studies of lipases from *Penicillium* sp. and *Rhizopus arrhizus* were used to predict enzyme specificity leading to fatty acid concentration (Mukherjee, Kiewitt, & Hills, 1993). It was reported that fatty acids with the first double bond from the carboxyl end as a cis-4 (22:6n-3), cis-6 (18:1n-12, 18:3n-6, 18:4n-6), or a cis-8 (20:3n-6) are strongly discriminated by the lipase (Mukherjee et al., 1993).

3.5. Preparation of PUFA concentrates by urea complexation

The per cent fatty acid composition obtained after urea complexation are illustrated in Table 2. Urea complexation of fatty acids was used to concentrate PUFA:s from SFAs and MUFA:s, where urea occludes straight-chain SFAs and MUFA:s in the hexagonal...
moiety, thus eliminating methylene-interrupted PUFAs. This is due to the non-uniformity in these molecules, caused by bends at the location of the olefinic bond (Chakraborty & Paulraj, 2007). Urea complexation resulted in the reduction in SFAs (2.4%) and MUFAs (15.7%) (Table 3). The total PUFAs after urea complexation was recorded to be 78.8%, mainly contributed by 20:5\text{n}-3, 18:3\text{n}-3, and 22:6\text{n}-3 (Table 3). In an earlier study, the fatty acids containing enriched 20:5\text{n}-3 (46.3%) and 20:4\text{n}-6 (3.9%) were recovered by partial hydrolysis using lipase purified from \textit{B. licheniformis}, and were further concentrated by urea complexation to 55.4 and 5.8%, respectively (Chakraborty & Paulraj, 2008b).

### 3.6. Argentated column chromatographic fractionation of n-3 PUFAs

The fatty ester profiles of the various solvent fractions obtained by argentation chromatography are shown in Table 3. The resolving capability of Ag-alumina is mainly attributed to reversible charge transfer complexation of Ag\(^+\) with \(-\text{C}==\text{C}\)- double bonds (Chakraborty & Paulraj, 2007). The first solvent fraction, i.e., n-hexane/Me\(_2\)O (99:1, v/v), was found to elute 16:1\text{n}-7 (24.6%) and 18:1\text{n}-9 (72.2%) amongst the MUFA methyl esters, and 18:2\text{n}-6 (0.3%) amongst PUFA methyl esters (Table 3). Elution of fatty acids with n-hexane/Me\(_2\)O (99:1, v/v) substantially eliminated the remainder of MUFAs (97.4%) from the urea concentrate. The product contains methyl esters 20:5\text{n}-3 (68.3 ± 2.2%), n-6's (2.6%), and other fatty acid esters (<1%) after using n-hexane/EtOAc (9:4, v/v). The second solvent fraction (n-hexane/Me\(_2\)O, 95:5, v/v) was eluted with a total of 23.8% MUFA methyl esters with 18:1\text{n}-9 and 16:1\text{n}-7 (Table 3). However, the predominant fatty ester eluted using this solvent system was found to be 18:3\text{n}-3 (56.1%), and 18:4\text{n}-3 (12.3%) was found to co-elute with it. The fractions eluted with n-hexane/Me\(_2\)O (95:5, v/v) were also substantially rich in 18:2\text{n}-6 (Table 3). Solvent fractions with 5% Me\(_2\)O/n-hexane contained 20:5\text{n}-3. The fatty acid 18:3\text{n}-3 was found to be eluted with n-hexane/CH\(_2\)OH.

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**Fig. 3.** A diagram showing differential hydrolytic resistance of n-3 (20:5\text{n}-3) and n-6 (20:4\text{n}-6) fatty acids towards \textit{B. circulans} lipase in a mixed triglyceride resulting in higher proportion of n-3 fatty acids in the triglyceride fraction. The n-3 terminal moiety with an olefinic part at the third carbon atom from the terminal methyl carbon atom hinders lipase-catalysed hydrolysis. In n-6 fatty acids, the n-6 terminal moiety without an olefinic bond induced lipase-catalytic hydrolysis of triglycerides, resulting in their reduced content in the hydrolysate fraction. The n-3 fatty acids, due to their lower affinity for lipase, remain in the native form. The triglyceride α-arachidonate-α,β-dieicosapentaenoate with one arachidonic acid (20:4\text{n}-6) and two eicosapentaenoic acid (20:5\text{n}-3) undergoes hydrolysis by lipase to form a fatty alcohol (α-hydroxypropyl-α,β-dieicosa-5,8,11,14,17-pentaenoate) with unhydrolysed 20:5\text{n}-3.
Me₂O (20:1, v/v) with a final purity of 56.1 ± 3.2%. The methyl ester of 22:6n-3, with an additional double bond, was found to co-elute (22:6n-3, 8.7 ± 0.4%) with 20:5n-3 in the fraction containing n-hexane/Me₂O (9:4, v/v) as eluting solvent system (Table 3). AgNO₃-TLC was used to elucidate the progress of purification of PUFAs. The uppermost band (Rₛ: 0.80–86) of SFAs, and the second band of MUFAs (Rₛ: 0.46–55) were apparent. FAME up to trienes (18:2n-6 and 18:3n-3) remained at the base of TLC chromatogram. These results are in agreement with the reported literature (Chakraborty & Paulraj, 2007).

### 4. Conclusions

The present study showed the changes in different classes of fatty acid and composition as a function of time-course (1–6 h) of hydrolysis of triglycerides from sardine oil by a lipase purified from *B. circulans* that may be a potential enzyme source for concentration of n-3 PUFAs of acyl chain length shorter than C20. The n-3 PUFAs (18:3n-3 and 20:5n-3) in the triglyceride mixture were found to increase proportionally with the progress of hydrolysis up to 3 h; after that their concentration plateaued as reaction time increased beyond 3 h, due to reduced selectivity of the lipase. It was found that ester bonds of n-3 fatty acid homologues were highly resistant to hydrolysis by lipase from *B. circulans*, resulting in their enrichment in fatty acid triglycerides. Structure–bioactivity relationship analyses revealed a direct relationship of hydrophobic and steric properties to lipase-catalysed hydrolytic effects on fatty acyl ester bonds of triglycerides. The urea complexation reaction raised 20:5n-3 and 18:3n-3 contents by 2.91-fold and 3.92-fold, respectively, thus further purification by argentated chromatography.

The notations are as indicated under Table 1. On the basis of the significance of treatments, LSD at the 5% level of significance (p = 0.05) was computed. Data are presented as mean values of three samples (means ± standard deviation). These values do not total 100% because minor fatty acids are not reported. ND implies non-detectable (or fatty acids present below 0.05%).

### References


