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Production of $n - 3$ polyunsaturated fatty acid concentrate from sardine oil by lipase-catalyzed hydrolysis

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Abstract

The production of $n - 3$ polyunsaturated fatty acids ($n - 3$ PUFAs) concentrate from oil extracted from Pacific sardines (*Sardinops sagax*) was studied using lipase-catalyzed hydrolysis. Commercially available microbial lipases, from *Candida Rugosa* (CR), *Candida cylindracea* (CC), *Mucor javanicus* (MJ), and *Aspergillus niger* (AN) were used for enzymatic hydrolyses with extracted sardine oil, run at 37 °C with constant stirring for 1.5, 3, 6, and 9 h. Fatty acid composition analysis by gas chromatography showed that the refined unhydrolyzed oil contained 26.86% of eicosapentaenoic acid (EPA) and 13.62% of docosahexaenoic acid (DHA) (wt/wt%). CR lipase was the most effective in concentrating $n - 3$ PUFA. Hydrolysis with 250 U CR lipase increased EPA concentration to a relatively constant level of 33.74% after 1.5 h. DHA levels were also significantly increased from 13.62% to 29.94% with 500 U after 9 h. Compared to CR and CC lipases, MJ and AN lipases resulted in low $n - 3$ PUFA concentration. Triacylglycerol levels decreased significantly as reaction time progressed.
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1. Introduction

Interest in fish consumption has been increasing recently due to the wide range of health benefits being discovered in long chain marine fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There is general consensus about the beneficial effects of fish consumption and the reduction of coronary heart disease (CHD). These include studies from over two decades ago when epidemiologists observed the low rate of coronary heart disease among Alaskan and Greenland Eskimos who consumed large amounts of fish (Nettleton, 1995). In both the US and Europe, several studies have also confirmed the positive effects of fish consumption in reducing CHD among diverse populations (Mozaffarian, Bryson, Lemaitre, Siscovick, & Burke, 2005). More recently a Japanese study involving more than 40,000 individuals showed a 40% reduction in CHD among those who ate fish more than four times per week (Iso et al., 2006). The American Heart Association has recommended that all individuals should eat fish, especially fatty fish, twice a week and those with documented CHD should eat a fish diet or take fish oil supplements that supply 1 g/day of EPA and DHA (Kris-Etherton, Harris, & Appel, 2002). There has also been research in the last decade showing the positive effects of fish oils on cognitive development and vision enhancement in newborns as well as young children (Colombo et al., 2004). The long chain fatty acids EPA and DHA have also been shown to contribute to the reduction of certain types of cancers, diabetes, mental health disorders and asthma (Alasalvar, Shahidi, & Quantick, 2002; Nettleton & Katz, 2005). Kalmijin, Van Boxtel, and Ocke (2004) showed that consumption of fatty fish high in EPA and DHA reduced the risk of impaired cognitive function in a study of middle-aged Europeans, while Morris et al. (2003) showed that

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elderly study participants had 60% less risk of developing Alzheimer's disease if they consumed fish one or more times per week.

With growing public awareness of the clinical benefits of EPA and DHA, various types of products and supplements have been developed. Sales of fish oil supplements have grown almost 10-fold over the last decade, reaching sales of $310 million in 2004 (Fiorillo, 2005). Most of these represent fish oil capsules made from various seafood sources including fish byproducts. Consumption of EPA and DHA in a concentrated form may be more effective than general fish oil itself because these concentrates contain less saturated fatty acid (SFA). Lipase-catalyzed enzymatic production of EPA and DHA concentrate from fish oil has shown potential in producing a high quality product due to the mild conditions (e.g., neutral pH and low temperatures) of the process (Breivik, Haraldsson, & Kristinsson, 1997). A consistently available source of high quality raw material is critical for production of $n-3$ polyunsaturated fatty acid ($n-3$ PUFA) concentrate. A new sardine (*Sagax sardinops*) fishery off the Oregon-Washington coast could provide a sustainable supply of the necessary raw material. In 2005 alone, 45,000 metric tons of sardines were landed in Oregon, the majority of which was sold for non-human food uses (McCrae & Smith, 2006). Previous study has shown that during peak harvest months these sardines have high lipid content (15–23%) with concomitant high levels of EPA and DHA. The objectives of this study were to: recover the lipids from sardines, produce EPA and DHA concentrates using lipase-catalyzed hydrolysis, and determine the optimal processing parameters for production of a fish oil concentrate.

2. Materials and methods

2.1. Sample preparation

Sardines were harvested by purse-seine fishing boats and pumped directly from the nets into refrigerated seawater holds on board the vessels followed by rapid cooling to 0–4 °C. The area of catch was approximately 40 km off shore from the mouth of Columbia River. Sardines were off-loaded at a processing plant in Astoria and transported to Oregon State University Seafood Laboratory in ice on the same day of capture. The head, viscera and backbone were removed from each fish, and skin-on fillets were obtained. The fillets were mixed together, minced by a vertical-cutter/mixer for 2 min and kept in vacuum-sealed bags at −30 °C for further analysis.

2.2. Extraction and purification of sardine oils

Sardine oil was extracted from minced sardine and refined by bleaching and deodorization according to Sathivel, Prinyawiwatkul, King, Grimm, and Lloyd (2003) with some modifications. Deionized water was added to the minced sardine in a 1:1 ratio, and the mixture was heated at 85 °C for 30 min. The lipid phase was removed from other particles by centrifuging at 2600*g* for 30 min followed by filtration under vacuum and then stored as crude oil at −80 °C until tested. The crude oil was refined by filtrating it under vacuum followed by neutralization with KOH. The extracted oils were bleached by adding 4% activated carbon and heating in a water bath at 70 °C for 10 min. The activated carbon and adsorbed impurities were removed from the oil by centrifugation at 2600*g* for 30 min. Partial deodorization of the bleached oil was carried out using a laboratory distillation unit with a round-bottom boiling flask (500 ml) having three outlets. One outlet was connected to a distillation column, the second outlet was connected to a vacuum pump, and the third outlet was connected to a thermometer. The boiling flask containing the bleached oil (100 ml) was heated to 100 °C for 30 min under vacuum (5 mm Hg). Next, the vacuum line was turned off, and the deodorized oil was transferred to a glass container where the air in the container was immediately replaced with N_2. Tertiary-butylhydroquinone (200 ppm) was added to the deodorized oil as an antioxidant with constant agitation with N_2. The oil was collected in 5 ml vials, the air in the vials was replaced by N_2 and the vials were stored as refined oil at −80 °C until further use. The initial amount of sardine flesh was 16.38 kg and the yields of crude and refined oils were 1.54 kg and 1.18 kg, respectively.

2.3. Determination of enzyme activities

Four commercially available microbial lipases were used for testing: *Candida Rugosa* (CR) lipase (Sigma–Aldrich Co., St. Louis, MO), *Candida cylindracea* (CC) lipase (Fluka Chemie AG, Buchs, Switzerland), *Mucor javanicus* (MJ) lipase (Aldrich Chemical Company, Inc., Milwaukee,WI), and *Aspergillus niger* (AN) lipase (Aldrich Chemical Company, Inc., Milwaukee, WI). The characteristics of these lipases are shown in Table 1. CR and CC lipases were non-specific lipases and MJ and AN lipases were 1,3-specific lipases. Enzyme activities were determined by the Japanese industrial standard method substituting sardine oil for olive oil (JIS, 1995). Free fatty acid (FFA) released by the hydrolysis reaction (20 min) were titrated against 0.5 N sodium hydroxide and the pH changes were monitored by adding 1% methanolic phenolphthalein solution. One unit of enzyme activity (U) was defined as the amount of enzyme that liberated 1 μmol of fatty acid per min at 37 °C.

2.4. Hydrolysis reaction

The hydrolysis of sardine oil by microbial lipases and separation of the $n-3$ PUFAs enriched fraction were carried out according to a modified procedure outlined in previous studies (Sun, Pigott, & Herwing, 2002; Wanasundara & Shahidi, 1998). Enzyme powder representing 250 U and 500 U per g oil of lipase with 8 ml of 0.1 N phosphate buffer at the optimum pH of each enzyme were mixed with

Table 1
Microbial lipases and their characteristics

Enzyme origin	Abbreviations	Optimum temperature (°C)	Optimum pH	Specificity	Activity (U)
Candida rugosa	CR	30–50	7.0	None	10,355
Candida cylindracea	CC	30–50	6.5	None	34,436
Mucor javanicus	MJ	30–45	7.0	1-, 3- $\gg$ 2-	2352
Aspergillus niger	AN	30–50	6.5	1-, 3- $\gg$ 2-	4741

refined sardine oil (2 g) and placed in a 25 ml flat-bottomed flask. The air in the flask was replaced by N_2, and the flask was capped with a glass lid covered by parafilm to minimize lipid oxidation. The mixture was agitated with a magnetic stir bar at 500 rpm. The hydrolysis reaction was maintained at 37 °C for 1.5, 3, 6, and 9 h, and lipase was inactivated by addition of 10 ml of 95% ethanol. A previously determined amount of KOH necessary to neutralize FFA released by hydrolysis was added to the mixture. Aliquots of 100 ml hexane and 50 ml deionized water were added to remove neutralized FFA with two additional water extractions. The hexane layer, including mono-, di- and triacylglycerols (MG, DG, and TG, respectively) with $n-3$ PUFAs, was collected and filtered through a bed of anhydrous sodium sulfate. Hexane was removed by a rotary evaporator at 45 °C for 30 min, and final $n-3$ PUFA concentrate was flushed with N_2 and stored at −80 °C until further use.

2.5. Determination of degree of hydrolysis

Degree of hydrolysis (DH) was determined by measuring the acid value of both unhydrolyzed and hydrolyzed oil at different treatment times (1.5, 3, 6, and 9 h) and concentrations as well as saponification value of unhydrolyzed oil according to American Oil Chemists' Society (AOCS) methods (AOCS, 1998). Blanks (no enzyme) were determined at each treatment. DH was calculated according to the following equation:

$$\mathrm{DH}(\%) = \frac{\text{acid value (hydrolyzed oil} - \text{blank at each condition)}}{\text{saponification value (original oil)} - \text{acid value (original oil)}} \times 100$$

Acid values indicate relative amount of free fatty acids released by hydrolysis reaction as measured by the mg of potassium hydroxide necessary to neutralize fatty acids in 1 g of sardine oil.

2.6. Determination of fatty acid composition by gas chromatography

Fatty acids in original sardine oil and final $n-3$ PUFA concentrate were converted into fatty acid methyl esters (FAME) according to AOCS method 991.39 (AOCS, 1998), and their composition was determined by gas chromatography (GC). A Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), equipped with a flame-ionization detector, capillary column (EC-wax, 30 m × 0.25 mm i.d.; split ratio, 100:1; Alltech, Deerfield, IL) was used for analyzing FAME. GC parameters were set as follows: injector and detector temperatures set at 250 °C and 270 °C, respectively; column temperature set at 50 °C, with gradual heating to 180 °C at a rate of 5 °C/min, followed by slow heating to 220 °C at a rate of 0.8 °C/min; and helium was used as a carrier gas. The fatty acid concentrations were calculated by comparison of their retention times with those of the reference standards (Supelco, Bellefonte, PA).

2.7. Thin-layer chromatography separation

The original sardine oil and final $n-3$ PUFA concentrate were separated into MG, DG, and TG fractions by thin layer chromatography (TLC). Samples were dissolved in hexane (10% solution, v/v) and an aliquot of 5 μl hexane-sample mixture was loaded onto a silica plate (K6 silica gel 60, Whatman Inc., Florham Park, NJ). Solvent development was applied with hexane/diethylether/acetic acid (60:40:2, volume basis), and the spots were visualized by spraying 5% sulfuric acid solution followed by drying at 100 °C on the hot plate for 30 min. TLC plates with dark brown spots were scanned (CanoScanLide 30, Canon USA, Inc., Lake Success, NY), converted to a bitmap file (8 bit gray scale, 100 dpi) and analyzed by a picture evaluation program (Image J: http://rsb.info.nih.gov/ij/) to obtain the chromatograph chart from each fraction. The standard curve was made by using commercially available TLC standard (Cat. No. 18-3A, Nu Check Prep, Inc., Elysian, MN), and each measurement was made by comparison with the standard.

2.8. Statistical analysis

Each hydrolysis reaction was run in duplicate and all analyses were run in triplicate. The results were presented as average and standard deviations. Statistical comparisons were made between treatments by analysis of variance and Tukey's test, and linear regression was applied using S-PLUS software. The results were presented in terms of p values ($p < 0.05$).

3. Results and discussion

3.1. Fatty acid composition in extracted sardine oil

Fatty acid compositions of crude and refined unhydrolyzed oil (wt/wt%) are shown in Table 2. The SFA fractions of crude and refined oils were 32.95% and 36.87%, respectively. The predominant fatty acid in both oils was 16:0, accounting for approximately 67% of each SFA fraction. The monounsaturated fatty acid (MUFA) fraction was 18.34% and 19.27% for crude and refined oil, respectively, with the predominant fatty acid being 16:1$n-7$ followed by 18:1$n-9$. The PUFA fractions of crude and refined oils were 48.29% and 43.17%, respectively. Total $n-3$ PUFAs (18:3$n-3$, 20:3$n-3$, 20:5$n-3$, and 22:6$n-3$) accounted for 96% of the PUFA fractions of both crude and refined oil, with high levels of 20:5$n-3$ (EPA) and 22:6$n-3$ (DHA). A study by Shirai, Terayama, and Takeda (2002) using *Sardinops melanostictus* caught in Japan showed SFA fractions of 34.3–41.4%, MUFA fractions of 15.1–22.4%, and PUFA fractions of 37.3–49.4%. These levels were in agreement with the present study, except that Shirai et al. (2002) reported a higher DHA concentration compared to EPA. Sardines caught in California (*Sardinops sagax caeruleus*) showed a similar ratio of EPA to DHA as in the present study; however, the California sardines had lower overall levels of EPA and DHA (Gamez-Meza et al., 1999). Sardines caught near Astoria showed higher amounts of $n-3$ PUFAs compared to other species such as *Sardina pilchardus W* caught in Spain (Beltran & Moral, 1991) and *Sardinops melanostictus* caught in Japan (Shirai et al., 2002).

A certain amount of $n-3$ PUFAs may be lost due to oil refining processes that involve high temperature treatments, such as bleaching and deodorization. A study by Sathivel et al. (2003) reported an approximate 19% reduction in the $n-3$ PUFAs of catfish viscera after oil refining processes including degumming, neutralizing, bleaching and deodorization where most of the reduction occurred at the deodorization step. In the present study, the fraction of total $n-3$ PUFAs decreased significantly by 9.43% following the refining steps. The greatest reductions in the PUFA fraction were observed with DHA (−3.06%) and EPA (−1.34%). There were slight increases in 14:0, 16:0 and 17:0 in the refined SFA fraction compared to the crude fraction and no significant difference in fatty acid composition of the MUFA fraction.

Table 2
Fatty acid profiles of crude and refined sardine oil (wt/wt%)

Fatty acid	Crude	Refined
C14:0	6.34 ± 0.41^a	7.38 ± 0.15^b
C16:0	22.2 ± 0.51^a	25.0 ± 0.28^b
C17:0	0.33 ± 0.01^a	0.36 ± 0.00^b
C18:0	4.06 ± 0.15^a	4.16 ± 0.03^a
Total SFA	33.0^a	36.9^b
C16:1$n-7$	9.58 ± 0.52^a	10.7 ± 0.12^a
C18:1$n-9$	5.23 ± 0.09^a	5.27 ± 0.06^a
C20:1$n-9$	2.87 ± 0.04^a	2.77 ± 0.03^a
C24:1$n-9$	0.66 ± 0.05^a	0.55 ± 0.02^a
Total MUFA	18.3^a	19.3^a
C18:2$n-6$	1.03 ± 0.03^a	0.29 ± 0.01^b
C18:3$n-6$	0.33 ± 0.02^a	0.34 ± 0.02^a
C18:3$n-3$	0.57 ± 0.01^a	0.62 ± 0.01^a
C20:2$n-6$	0.22 ± 0.01^a	0.20 ± 0.00^a
C20:3$n-6$	0.20 ± 0.01^a	0.20 ± 0.00^a
C20:3$n-3$	0.78 ± 0.01^a	0.77 ± 0.01^a
C20:4$n-6$	0.28 ± 0.04^a	0.27 ± 0.04^a
C20:5$n-3$	28.2 ± 0.17^a	26.9 ± 0.17^b
C22:6$n-3$	16.7 ± 0.68^a	13.6 ± 0.16^b
Total PUFA	48.3^a	43.2^b
Total $n-3$ PUFA	46.2^a	41.9^b

Values in the same row labeled with a different superscript letter are significantly different ($p < 0.05$).

3.2. Enzyme activity and degree of hydrolysis

CC lipase showed the highest enzyme activity (34,436 U/g lipase) among all lipases followed by CR lipase (10,355 U/g lipase), while AN (2352 U/g lipase) and MJ (4741 U/g lipase) lipases showed much lower levels (Table 1). These results demonstrated that the non-specific lipases used in this study had higher activity than the 1,3-specific lipases in the presence of sardine oil.

DHs of sardine oils from different lipases, concentrations and reaction times are shown in Fig. 1. In general, sardine oil was hydrolyzed rapidly during the initial 1.5 h. Afterwards, hydrolysis was more gradual, and only minor increases were shown throughout the 3, 6, and 9 h time periods. The highest DH (78.40%) was shown by CR 500 U lipase treatment at 9 h followed by CR 250 U

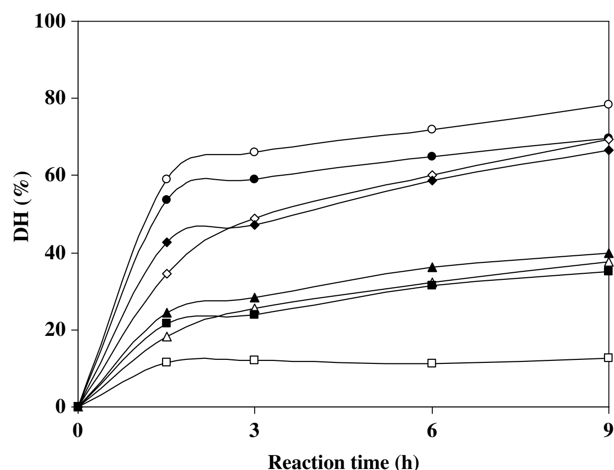

Fig. 1. Degree of hydrolysis (%) of hydrolyzed sardine oil by lipases at 37 °C (individual incubations for each lipase at each reaction time). ○, CR 250 U; ●, CR 500 U; ◇, CC 250 U; ♦, CC 500 U; △, MJ 250 U; ▲, MJ 500 U; □, AN 250 U; ■, AN 500 U.

(69.70%) and CC 250 U (69.33%) at 9 h. The lowest DH (12.68%) was shown by 250 U AN lipase after 9 h of hydrolysis. In general, the DH was higher in oil hydrolyzed by non-specific lipases (CR and CC) compared with 1,3-specific lipases (AN and MJ), which is in agreement with other studies on cod liver oil, salmon viscera oil and menhaden oil (Hoshino, Yamane, & Shimazu, 1990; Sun et al., 2002; Wanasundara & Shahidi, 1998). Wanasundara and Shahidi (1998) showed that the DH of seal blubber oil treated with 200 U CC lipase after 9 h was greater than 70%, while menhaden oil showed a lower DH. They reported that the difference was due to the presence of higher amounts of PUFA in menhaden oil than seal blubber oil. However, refined unhydrolyzed sardine oil in the present study had a higher DH and had higher amounts of EPA and DHA (26.86% and 13.62%, respectively) than seal blubber oil (4.66% EPA and 7.58% DHA) and menhaden oil (2.40% EPA and 10.06% DHA).

3.3. Fatty acids profiles after hydrolysis

All lipases caused changes in fatty acid composition, but in varying amounts. Ideally, enzymatic activity followed by FFA removal will increase EPA and DHA concentrations while reducing SFA and MUFA. Fig. 2 shows the changes in 16:0, 16:1$n-7$, EPA, and DHA content in the final product obtained after lipase-catalyzed hydrolysis followed by FFA removal with neutralization. Linear regression analysis showed that EPA content was affected by enzyme and enzyme concentration, while DHA content was affected by enzyme and reaction time. With CR 250 U, EPA content significantly increased from 26.87% to 33.74% after 1.5 h and remained at relatively constant levels after 3, 6 and 9 h (33.45%, 34.45% and 33.17%, respectively). A similar tendency was observed with CR 500 U except that EPA was present at slightly lower levels, ranging from 30.69% to 33.06%. DHA levels also increased significantly after 1.5 h, from an original of 13.63% to 23.12% with 250 U and to 23.78% with 500 U. Minor increases in DHA concentration from 24.27% to 28.02% with 250 U and 26.87–29.94% with 500 U occurred as hydrolysis continued to 9 h, although there was no significant difference between 1.5 h and longer reaction times. The highest total $n-3$ PUFA fraction (63.86%) was found in the oil hydrolyzed with 250 U CR for 6 h (data not shown). The 16:0 content decreased significantly from 24.99% to 17.07% after 1.5 h and gradually decreased to 13.24% after 6 h with CR 250 U; the same pattern was observed with CR 500 U. Also, levels of 16:1$n-7$ decreased significantly after 1.5 h from 10.68% to 5.11% and 5.78% with 250 U and 500 U, respectively; however the levels remained relatively constant for the rest of the hydrolysis reaction time (up to 9 h).

Hydrolysis with CC lipase also resulted in high concentrations of $n-3$ PUFAs in the final products. EPA content increased significantly after 1.5 h from 26.86% to 31.91% and 31.19% with 250 U and 500 U, respectively. DHA content also increased significantly after 1.5 h from 13.62% to 22.65% and again after 6 and 9 h (to 26.16% and 26.54%,

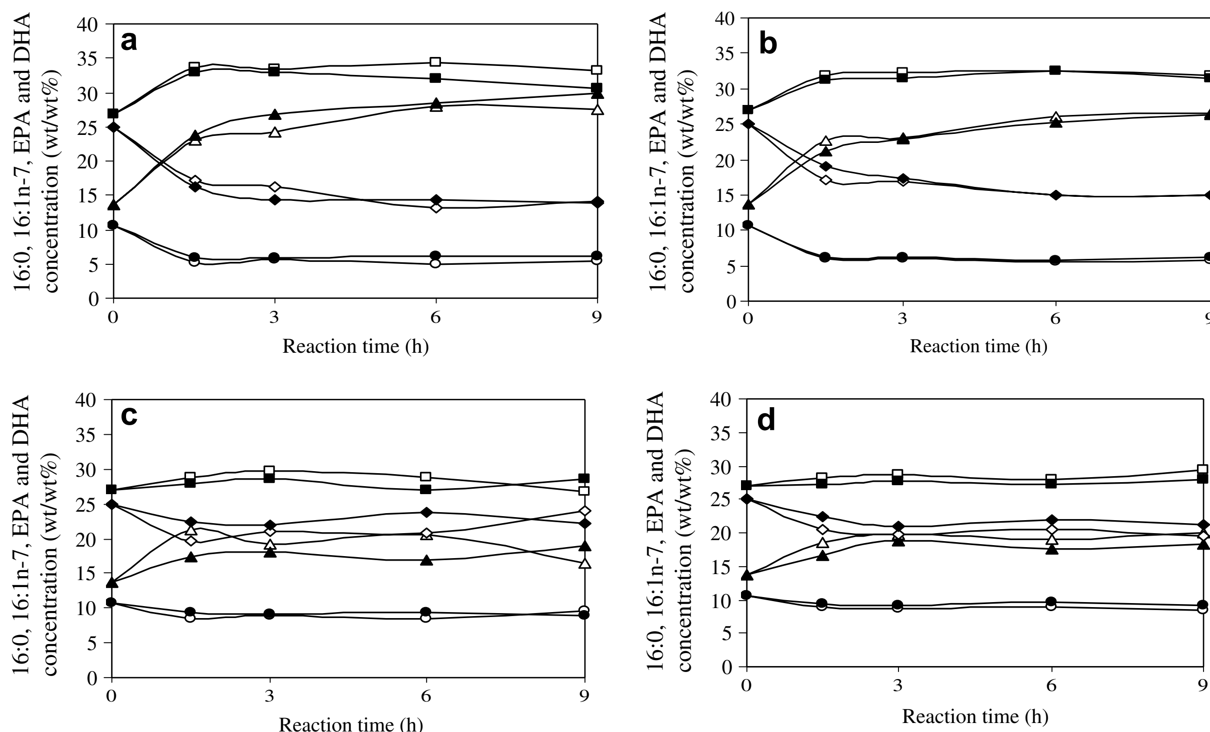

Fig. 2. Changes in 16:0, 16:1$n-7$, EPA, and DHA concentration (wt/wt%) in final $n-3$ PUFA concentrate with lipases from (a) CR, (b) CC, (c) MJ and (d) AN during hydrolysis at 37 °C. ◇, 16:0 with 250 U; ♦, 16:0 with CR 500 U; ○, 16:1$n-7$ with 250 U; ●, 16:1$n-7$ with 500 U; □, EPA with 250 U; ■, EPA with 500 U; △, DHA with 250 U; ▲, DHA with 500 U.

respectively) with 250 U ($p < 0.05$). With 500 U, DHA increased significantly from 13.62% to 21.14% after 1.5 h and then increased significantly at 9 h (26.32%). These EPA and DHA changes were concomitant with significant decreases of both 16:0 and 16:1$n-7$ after 1.5 h (from 24.99% to 17.19% and from 10.68% to 6.03% with 250 U, and from 24.99% to 19.08% and from 10.68% to 6.15% with 500 U, respectively).

AN lipase was found to be less effective at increasing $n-3$ PUFA concentration compared with CR and CC lipases. Oil hydrolyzed by AN lipase exhibited only a minor increase in DHA content and no significant changes in EPA content. This result was in agreement with Hoshino et al. (1990). Although the changes in DHA concentration were much smaller than changes resulting from either CR or CC lipases, DHA levels did increase slightly from 13.63% to 18.46% with 250 U after 1.5 h and to 18.72% with 500 U after 3 h. However, at 6 and 9 h, DHA levels remained relatively constant with no significant differences. Minor decreases in 16:0 content were observed after 1.5 h with 250 U (from 24.99% to 20.41%) and after 3 h with 500 U (from 24.99% to 20.88%). There were also reductions in 16:1$n-7$ levels after 9 h with 250 U (from 10.68% to 8.44%) and after 3 h with 500 U (from 10.68% to 9.11%). Despite the low activity of AN lipase in concentrating $n-3$ PUFAs in sardine oil, it did cause slight changes in levels of other fatty acids, such as 18:2$n-6$, which increased from 0.19% to 1.06% and 1.11% (250 U and 500 U, respectively) after 1.5 h. The 20:3$n-3$ content also increased slightly after 9 h, going from 0.78% to 0.92% (data not shown).

Among all lipases, MJ lipase showed the lowest effect on $n-3$ PUFAs concentration. No significant increases were found in EPA content by treatment with either 250 U or 500 U of this lipase. Only minor increases in DHA were observed after 1.5 h (from 13.62% to 21.34%) with 250 U and there was no significant change in DHA content with 500 U. Levels of 16:0 and 16:1$n-7$ decreased slightly with 250 U after 1.5 h, from 24.99% to 19.70% and from 10.68% to 8.45%, respectively; however, there were no significant differences with 500 U.

This study showed that lipase-catalyzed hydrolysis, especially with CR and CC lipases, resulted in successful concentration of both EPA and DHA with different efficiencies depending on conditions. In general, there were significant increases in levels of both EPA and DHA after 1.5 h of hydrolysis with just slight increases afterwards, suggesting 1.5 h of reaction time may be sufficient enough to achieve fairly high levels of $n-3$ PUFA concentration in the final product. A further benefit is in the reduction of SFA as well as MUFA fractions, particularly 16:0 and 16:1$n-7$. This indicates an ability of the CR and CC lipases to discriminate SFAs and MUFAs from EPA and DHA in fish oils, most likely due to the reduced steric hindrance observed with SFAs and MUFAs when linked to a glycerol backbone (Gamez-Meza et al., 2003).

The molecular conformation of *cis* carbon–carbon double bonds in PUFAs, particularly EPA and DHA, causes steric hindrance and subsequent bending of the fatty acid chains, bringing the terminal methyl groups very close to the ester bonds. Because of this steric hindrance effect, enzymatic active sites cannot reach the ester-linkages of these fatty acids with their glycerol backbones, thereby protecting EPA and DHA from lipase-catalyzed hydrolysis. However, this does not occur with the relatively straight chains of SFAs and MUFAs, and therefore hydrolysis is not hindered (Carvalho, Campos, D'Addio Noffs, Bastos, & De Oliveira, 2002; Shahidi & Wanasundara, 1998). Also, it has been suggested that TGs without EPA and DHA are hydrolyzed in the first phase, and TGs with EPA and DHA are hydrolyzed later, indicating that the lipase recognizes the whole molecular structure, not only its ester bonds (Hoshino et al., 1990).

In this study, non-specific lipases showed a higher ability to concentrate EPA and DHA than 1,3-specific lipases, which is in agreement with other studies (Carvalho et al., 2002; Sun et al., 2002). One reason for the high efficiency of the non-specific CR lipase in the production of an $n-3$ PUFA concentrate may be found in its chainlength selectivity. CR lipase is known to possess fatty acid chain-length selectivity, showing higher activity with relatively short-chain fatty acids such as C18 or below (McNeill, Moore, & Ackman, 1996). Additional possible reasons for higher efficiency of non-specific lipases might be lipase regiospecificity and/or original fatty acid profiles of fish oil (Hoshino et al., 1990; McNeill et al., 1996). Preferential fatty acid hydrolysis could also depend on fish species, which may have varied positional arrangements of PUFAs. For example, the non-specific CR and CC lipases were not able to increase EPA content in salmon oil (Linder, Fanni, & Parmentier, 2005), salmon viscera oil (Sun et al., 2002) or Brazilian sardine oil (Carvalho et al., 2002), but did cause an increase in EPA content in seal blubber oil (Wanasundara & Shahidi, 1998) and in Pacific sardine oil (present study). Also, 1,3-specific lipase, which was found to be ineffective at $n-3$ PUFA concentration from sardine oil in this study, increased EPA and DHA levels in anchovy oil (Ustun, Guner, Arer, Turkay, & Erciyes, 1997).

In the case of fish oil, the second position of the glycerol moiety is usually more enriched with $n-3$ PUFAs (Bornscheuer, 2000), although this may vary depending on species (Gamez-Meza et al., 1999). For example, stereospecific analysis has revealed that EPA is preferentially located in position 3 of the glycerol backbone in sardine oil (Ando, Ota, Matsuhira, & Yazawa, 1996). The 1,3-specific lipases possess stereospecificity with respect to the 1- and 3-positions of the acylglycerols, hydrolyzing the fatty acids at these positions easier than at the 2-position, resulting in 2-MGs and a mixture of DGs and fatty acids (Uhlig, 1998). If the 2-position is high in EPA and DHA, 1,3-specific lipase should be more effective at concentrating $n-3$ PUFAs; however, if SFA or MUFA are located at the 2-position and PUFAs are located at the 1- and 3-positions

of triacylglycerols, hydrolysis with 1,3-specific lipase would most likely result in low $n-3$ PUFA levels in the final product (Sun et al., 2002). This could partially explain why 1,3-specific lipase was less effective at $n-3$ PUFA concentration compared to non-specific lipases in this study.

3.4. Changes in mono-, di- and triacylglycerols distribution and acid values after hydrolysis

The changes in levels of MG, DG, and TG as well as acid values are shown in Figs. 3 and 4. The unhydrolyzed, refined sardine oil contained 86.20% TG, 13.40% DG, 0.51% MG, and had an acid value of 2.06. TG levels were significantly less in all final $n-3$ PUFA concentrate compared to the original sardine oil. TG levels were significantly reduced from 86.20% to 70.46% with 250 U after 1.5 h and gradually decreased to 65.94% after 9 h, although there was no significant difference between 1.5 h hydrolysis and longer reaction times on TG levels. CR lipase at 500 U showed a similar trend as at 250 U, and TG levels decreased from 86.20% to 70.31% at 1.5 h. DG levels increased from 13.40% to 26.89% after 1.5 h and again (to 32.33%) after 9 h hydrolysis ($p < 0.05$). MG levels also increased after 1.5 h treatment from 0.51% to 2.08% at 250 U, with constant levels at longer reaction times. Acid values of oil hydrolyzed with CR lipase increased from 2.06 to 83.61 and 92.11 after 1.5 h and gradually increased to 108.22 and 122.15 after 9 h by CR lipase at 250 U and at 500 U, respectively. With CC lipase, TG levels were significantly reduced from 86.20% to 66.09% after 1.5 h by 250 U and the levels gradually decreased to 63.38% at 9 h. DG levels increased from 13.40% to 30.67% after 1.5 h and again increased to 33.35% after 9 h with 250 U, and the same tendency was observed with 500 U. MG levels increased from 0.51% to 3.75% after 1.5 h and remained constant for the rest of the hydrolysis time with 250 U. Acid values also increased from 2.38 to 48.92 after 1.5 h and gradually increased up to 104.83 after 9 h. Hydrolysis with 500 U showed similar acid values as with 250 U (61.58 after 1.5 h, with a gradual increase up to 100.71 after 9 h).

In the case of the MJ lipase, TG levels decreased significantly to from 86.20% to 70.90% after 1.5 h and again to 60.57% after 6 h, with an increase in DG content after 1.5 h (from 13.40% to 24.53%) and after 9 h (from 13.40% to 33.75%), as well as an increase in MG levels after 1.5 h (from 0.51% to 4.22%) and after 9 h (from 0.51% to 5.70%). The acid values increased from 2.06 to 70.66 and 61.99 after 9 h (250 U and 500 U, respectively), and were much lower than in oils hydrolyzed with non-specific lipases.

With AN lipase at 250 U, changes in fatty acid fractions showed somewhat different patterns and had the lowest acid values among all treatments. However, acid values did increase somewhat, going from 2.06 to 26.11 and to 31.26 (at 250 U and 500 U, respectively) after 1.5 h. TG

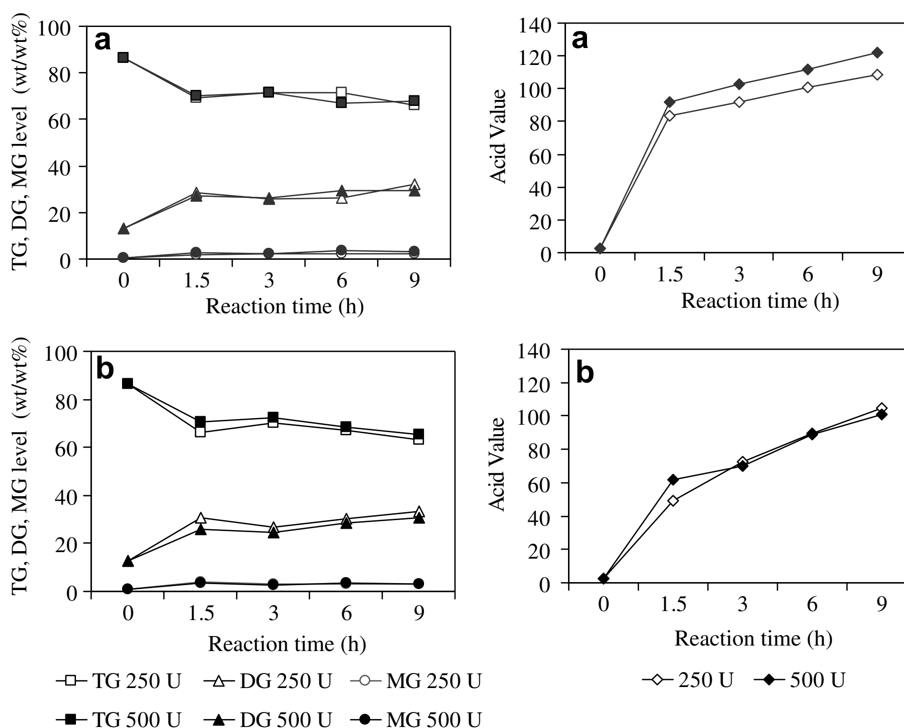

Fig. 3. Changes in TG, DG, and MG fractions of final $n-3$ PUFA concentrate, and acid values in hydrolyzed sardine oil with lipase from (a) CR and (b) CC during hydrolysis reaction at 37 °C. □: TG, 250 U; ■: TG, 500 U; △: DG, 250 U; ▲: DG, 500 U; ○: MG, 250 U; ●: MG, 500 U; ◇: acid value, 250 U; ♦: acid value, 500 U.

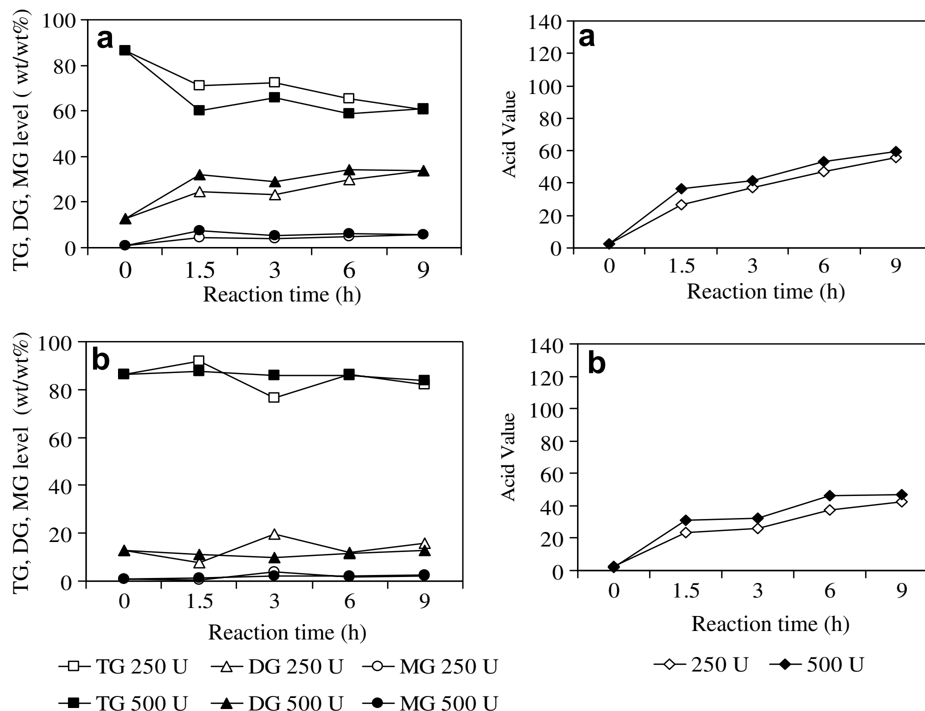

Fig. 4. Changes in TG, DG and MG fractions of final $n-3$ PUFA concentrate, and acid values in hydrolyzed sardine oil with lipase from (a) MJ and (b) AN during hydrolysis reaction at 37 °C. □: TG, 250 U; ■: TG, 500 U; △: DG, 250 U; ▲: DG, 500 U; ○: MG, 250 U; ●: MG, 500 U; ◇: acid value, 250 U; ♦: acid value, 500 U.

fractions decreased slightly, with an increase in DG and MG fractions, but fewer changes were observed compared to treatments with all other enzymes.

4. Conclusions

Non-specific lipases used in this study were significantly better than 1,3-specific lipases at concentrating $n-3$ PUFAs from sardine oil. Hydrolysis with CR lipase and CC lipase followed by FFA removal resulted in improved total $n-3$ PUFAs while AN and MJ lipases had little effect on concentrating $n-3$ PUFAs in sardine oil. It should be noted that $n-3$ PUFA levels increased significantly after 1.5 h, after which point only slight increases were observed. Thus, for commercial production runs, which depend on efficiency and through-put, 1.5 h might be the optimum reaction time for producing $n-3$ PUFA concentrates. The majority of the concentrated product had a relatively high amount of TG fraction even after 9 h hydrolysis. Overall, analysis showed that 250 U of lipase performed equal or better than 500 U of lipase, maintaining high TG levels and increasing $n-3$ PUFAs content in the final concentrated products. Sardines caught in the Oregon-Washington coastal area were shown to be a good source of fish oil, containing high levels of $n-3$ PUFAs, and lipase-catalyzed hydrolysis was demonstrated to be a feasible method for concentration of $n-3$ PUFAs from sardine oil for use in nutraceuticals and other products.

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