CHAPTER 2

TWO-STEP PROCESS ENZYMATIC ENRICHMENT OF *n*-3 POLYUNSATURATED FATTY ACIDS FROM FISH OIL

2.1 Abstract

Synthesis of polyunsaturated fatty acid-rich fish oil by a two-step enzymatic method that consisted of hydrolysis of tuna oil and selective esterification of resulting free fatty acids (FFA) to enrich the polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was studied. Refined tuna oil used in this study contained EPA and DHA 6.42 and 28.18%, respectively. Three kinds of immobilized lipases (Lipase PS; *Pseudomonas* sp.*;* Lipase AK; *Psuedomonas fluorescence* and Lipase D; *Rhizopus delemar*) were used. The results showed that immobilized Lipase D and Lipase PS provided the highest content of FFA above 80 wt% but hydrolysis with immobilized lipase D (100 U/g mixture, 40 °C for 24 h) provided the highest FFA (>80%) in the hydrolyzed tuna oil and also gave the highest degree of EPA and DHA (8.31 and 34.74% in FFA mixture). The FFA mixture was separated by saponification and solvent extraction and was further enriched in PUFA by selective esterification of hydrolyzed FFA mixture with long chain alcohol by these immobilized lipases. The immobilized lipase AK (100 U/g mixture, 50 °C for 24 h.) could esterified saturated fatty acids with octanol with good enrichment of PUFA (10.31% EPA and 50.74% DHA in FFA mixture) in the FFA mixture. Separation of saturated and less unsaturated free fatty acid from FFA mixture by urea complexation in an ethanolic solution (20 wt% urea at 4 °C) showed that yield of PUFA was increased with high content of EPA and DHA of 19.50 and 56.21%, respectively.

2.2 Introduction

Interest in fish oil has been linked to the presence of two long-chain polyunsaturated fatty acids (PUFA): *cis*-5, 8, 11, 14, 17-eicosapentaenoic acids (EPA) and *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA). DHA is important for brain and eye development (Svennerholm, 1968; Jonzo *et al.,* 2000). Ingesion of *n*-3 PUFA may prevent coronary diseases (Kinsella and Kella, 1988; Jonzo *et al.,* 2000). Medical studies clearly indicate that these fatty acids have beneficial health aspects, such as improved cardiovascular and immune functions and reduction of cancer, diabetes, and high blood pressure (Carroll, 1986; Simopoulos, 1991). Recent clinical results have demonstrated that dietary intake of 5.5 g of *n*-3 PUFA per week from seafood was linked to a 50% reduction in the risk of primary cardiac arrest (Siscovick *et al.,* 2005).

Consequently, oil containing *n*-3 PUFA has been in high demand for pharmaceutical and dietetic purposes. Many investigations have been actively undertaken in search of methods for concentrating *n*-3 PUFA from marine oils.

However, *n*-3 PUFA are highly labile and sensitive to heat and oxidation. Their all-*cis n*-3 structure is prone to partial destruction by oxidation, double- bound migration, *cis*-trans isomerization, or polymerization (Shimada *et al*., 1997a). Modification of lipids from marine oil normally involves a lipase-catalyzed process. First, the application of lipases as biocatalysts in processes such as fat hydrolysis (Knezevic *et al.,* 1998; Rice*,* 1998), triacylglycerol modification (Moore and McNeill, 1996; Schmid *et al.,* 1998; Akoh, 1995), and ester synthesis (Ward *et al.,* 1997; Shimada *et al*., 1999) is attractive because lipase efficiency is high and only a low amount of catalyst is required especially when immobilized. Lipases may be immobilized on various support materials. From a marketing view, immobilization technology offers many advantages such as the ability to reuse the catalyst, an easy separation of reaction products by the enzyme by filtration (Lokotch *et al.,* 1989) and a great potential to run continuous processes on a large scale (Rice, 1998; Garcia *et al.,* 1992; Malcata *et al.,* 1990). Secondly, lipase-catalyzed reactions are carried out under mild conditions and lead to the formation of specific compounds, which are easily isolated without molecular distillation in comparison to equivalent chemical processes, which usually occur at higher temperatures. Some lipases are recognized to have little reactivity with PUFA. Concentrates of both EPA and DHA may be

prepared by selective hydrolysis of fish oils by using lipases, which discriminate against *n*-3 PUFA (Hoshino *et al.,* 1990; Shimada *et al.,* 1995; Wanasundara and Shahidi, 1998) or by selective esterification (Hills *et al.,* 1990; Shimada *et al.,* 1997a; 1997b). Recently Shimada *et al*. (1995; 1997a) described the purification of DHA by selective esterification of the FFA from tuna oil with various alcohols catalyzed by *Rhizopus delemar* lipase. Lipase-assisted *n*-3 PUFA enrichment of fish oil may also be obtained by transesterification reactions (Haraldsson *et al.,* 1997). Therefore, it is convenient to prepare *n*-3 PUFA concentrates as FFA after enzymatic hydrolysis of marine oils, such as tuna oil.

In this study, enrichment of free fatty acids with *n*-3 PUFA according to Shimada *et al*. (1997a; 1997b) by immobilized lipases was carried out by two-step processes. In the first step; tuna oil is subjected to hydrolysis reaction catalyzed by enzyme lipase yielding the *n*-3 PUFA in FFA which were esterified in second step with long chain alcohol by enzyme lipases to achieve the highest concentration of *n*-3 PUFA in FFA. Moreover, the separation of saturated and less unsaturated FFA from FFA by urea complexation was also studied.

2.3 Materials and Methods

2.3.1 Materials

Crude tuna oil, with water content of 4.7%, was provided from Chotiwat Industrial Co. Ltd (Hat Yai, Thailand). The crude oil was obtained from skipjack tuna heads by a conventional pressing method. The refined oil was achieved through degumming, neutralization, bleaching, and deodorizing according to Rungsilp (1998). Refined tuna oil was stored under nitrogen at -20 °C in dark amber glass container until use. Lipase AK, from *Pseudomonas fluorescens*, Lipase PS; *Pseudomonas* sp. and Lipase D; *Rhizopus delemar* with water content of 0.04, 0.07 and 0.10 wt%, respectively, were a gift from Amano (Nagoya, Japan). The characteristic of immobilized lipases and their activity are shown in Table 10 and 11. Microporous polypropylene powder, Accurel EP-100 (particle size < 400 μm) was a gift from Akzo Nobel Membrana (Obernburg, Germany). All other chemicals and solvents used were of reagent grade or analytical grade.

Table 10. Activity, protein content and specific activity of different lipases

Commercial name	Activity (U/mg)	Protein content (mg protein/mg)	Specific activity (U/mg protein)
Lipase PS	6.40	0.07	96.83
Lipase AK	10.74	1.91	5.62
Lipase D	406.92	1.10	370.47

Table 11. Characteristic of the immobilized lipases

Source of lipase	Commercial name	Carrier	Immobilized activity	Protein binding

			(U/mg imm. enz.)	(%)
Psuedomonas sp.	Lipase PS	EP-100	0.94	18.80
Psuedomonas fluorescens	Lipase AK	EP-100	0.51	67.07
Rhizopus delemar	Lipase D	EP-100	1.45	3.56

2.3.2 Preparation of the immobilized lipases

Accurel EP-100 (10 g) was added to 100 mL of 0.1M phosphate buffer (pH 7) containing approximately 100 U/mL of lipases (Lipase AK, PS and D) and the mixture was stirred with a magnetic bar at 100 rpm for 30 min. Afterward, 100 ml of 0.1M phosphate buffer (pH 7) was added and the suspension was filtered through a Buchner funnel by vacuum. The immobilized enzyme was washed with 100 mL of buffer to remove the soluble enzyme.

The water activity of the immobilized enzyme was adjusted according to the method of Lee and Rhee (1993) and incubated separately in desiccator to obtain a defined initial water activity. Equilibration was performed for at least 16 h at 25 °C.

2.3.3 Hydrolysis of tuna oil

Hydrolysis of tuna oil was performed in the reaction mixture containing fish oil (15 g) mixed with water (15 g) and immobilized lipases (50 U/ g mixture) by shaking at 500 rpm and 40 °C. The final reaction mixture was filtered by Whatman No.1 filter paper connected with a vacuum pump to remove the immobilized lipase. The composition of filtrate was determined by TLC/FID (Shimada *et al.,* 1997b). After that, the FFA from the mixtures was extracted by solvent extraction according to Senanayake and Shahidi (1999) and Wanasundara and Shahidi (1998). The FFA mixture obtained from this step called hydrolyzed tuna oil and the composition of FA was determined by GC/FID.

2.3.4 Extraction of FFA from the mixture

Preparation of FFA mixture from the hydrolyzed tuna oil was carried out according to Senanayake and Shahidi (1999) and Wanasundara and Shahidi (1999) with slight modification. The mixture (25 g, treated with 200 ppm butylhydroquinone, BTHQ) was saponified using a mixture of KOH (5.75 g), water (11.0 ml) and 95% aqueous ethanol (66 ml) for 1 h at 62 °C under nitrogen condition. After saponification, distilled water (50 ml) was added to the saponified mixture and the unsaponifiable matter was extracted with 2×100 ml of hexane and discarded. The saponifiable matter in the aqueous layer was acidified to pH 1.0 with 6 N HCl and the FFA were extracted into 50 ml of hexane. The hexane layer containing free fatty acids was then dried over anhydrous sodium sulfate and the solvent was removed using a vacuum rotary evaporator at 40 °C. The FFA mixture was stored at -20 °C under nitrogen until use.

2.3.5 Selective esterification of fatty acids with long-chain alcohol

The FFA mixture obtained from hydrolyzed tuna oil, was esterified with octanol (1:3 mol/mol) (4 g), water (1 g) and immobilized lipase (100 U/g mixture) by shaking at 500 rpm and 30 °C for 48 h. The final reaction mixture was filtered by Whatman No.1 filter paper connected with a vacuum pump to remove the catalyst. The composition of the filtrate was determined by TLC/FID (Shimada *et al.,* 1997b).

2.3.6 Urea complexation

The separation of PUFA from the FFA mixture of the hydrolyzed tuna oil was carried out using urea-fatty acid adducting formation according to Senanayake and Shahidi (1999); Wanasundara and Shahidi (1999) and Zuta *et al*. (2003). Free fatty acids (10 g) were added under constant stirring to a hot (60 °C) solution of 30 g urea in 150 ml of 95% aqueous ethanol. The solution was heated and stirred until clear, then it was allowed to crystallize at room temperature for 5 h and kept at 4 °C for 24 h for further crystallization. The formed crystals were separated from the liquid by vacuum filtration. The filtrate was diluted with an equal volume of

water and acidified to pH 4-5 with 6 N HCl and equal volume of hexane was subsequently added and the mixture was stirred for 1 h, then transferred to a separatory funnel. The hexane layer containing free fatty acids was separated from the aqueous layer and washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulfate. The solvent was subsequently removed at 40 °C using a vacuum rotary evaporator.

2.3.7 Analysis of acylglycerols by TLC-FID

The components of oil phase were analyzed with a thin-layer chromatography with flame ionization detector (TLC/FID)(IATROSCAN MK5, Iatron Laboratories Inc., Tokyo, Japan) for the content of TAG, 1,2(2,3)-DAG, 1,3-DAG, MAG and free fatty acids (FFA) (Kaewthong and H-Kittikun, 2004). The samples diluted in chloroform/methanol (2.0:1.0 v/v) was spotted onto the chromarod and developed for 35 min in a mixture of benzene/chloroform/acetic acid (50:20:0.7, v/v/v) as developing solvent. After developing and drying, the rods were subjected to scanning with FID. Standards were used to identify the peaks. The peaks areas were normalized and used for evaluation of reactions. Triplicate analysis was conducted and the average was used to be reported in this paper.

2.3.8 Analysis of fatty acid compositions

The fatty acid compositions of acylglycerol species were determined by converting into fatty acids methyl esters followed by GC analysis. After evaporating excessive solvent of the sample, the mixture was applied to normal TLC-plate with silica gel and developed in benzene/chloroform/acetic acid (50:20:0.7, v/v/v). After drying, the MAG band was scraped off and methylated with 0.5% NaOH in methanol (1000 μL), for 10 min at 60 °C. The methyl

esters were extracted with *n*-hexane (300 μL) for 1 min. The *n*-hexane layer was washed with 200 μL distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with a Perkin-Elmer Autosystem XL-GC gas chromatograph (Perkin-Elmer Corporation, Norwalk, CT) on a FFFAP column (PERMABOND-FFFAP DF-0.25, 25m×0.25mm i.d., MACHEREY-NAGEL, Germany). The carrier gas used was helium set at a flow rate of 0.5 mL/min (15 *psi*) and operated in a spit ratio of 50:1. The temperature was started from 150 °C for 0.50 min and increased at the rate of 4 °C/min to 170 °C, followed with the rate of 5 °C/min to 195 °C, and further with the rate of 10 °C/min to and 215 °C the temperature was kept at 215 °C for 14 min. Injector and detector temperatures were 250 °C (Joseph and Ackman, 1992). Response factors were determined using a standard mixture of fatty acid methyl esters. Duplicate analysis was carried out for all the analysis and the average was used in the paper.

2.3.9 Statistical analysis

The SPSS program analysis was used for data analysis (SPSS, 1989-2001). Analysis of variance and t-test were used to evaluate the significance and difference of data. Values were considered significant at $P < 0.05$ level.

2.4 Results and discussion

2.4.1 Preparation of tuna oil and determination of compositions of glyceride species and fatty acids

The refined tuna oil was obtained after the refining processes which yield of 30% based on crude oil. The refined oil was stored under nitrogen at -20 °C in dark amber glass container until use. The refined oil that preserved in amber bottle under nitrogen gas could prevent the auto oxidation (Stansby, 1990; Xu *et al.,* 2000). Table 12 showed that tuna oil contained 99.32 wt% of triacylglycerol (TAG) and the main fatty acids contents are palmitic acid, stearic acid, oleic acid, EPA and DHA (Table 13). The contents of EPA and DHA in the refined

oil were 6.42 and 28.18 wt%, respectively. Shimada *et al.* (1994), Wongsakul *et al*. (2004) and Klinkesorn *et al.* (2004) also found high amount of EPA and DHA in the refined tuna oil.

Table 12. Glyceride contents (% peak area) of tuna oil

Compositions	**%**
Triacylglycerols (TAG)	99.32
Diacylglycerols (DAG)	0.32
Monoacylglycerols (MAG)	0.00
Free fatty acids (FFA)	0.36

Table 13. Fatty acid compositions (% peak area) of crude tuna oil and refined tuna oil

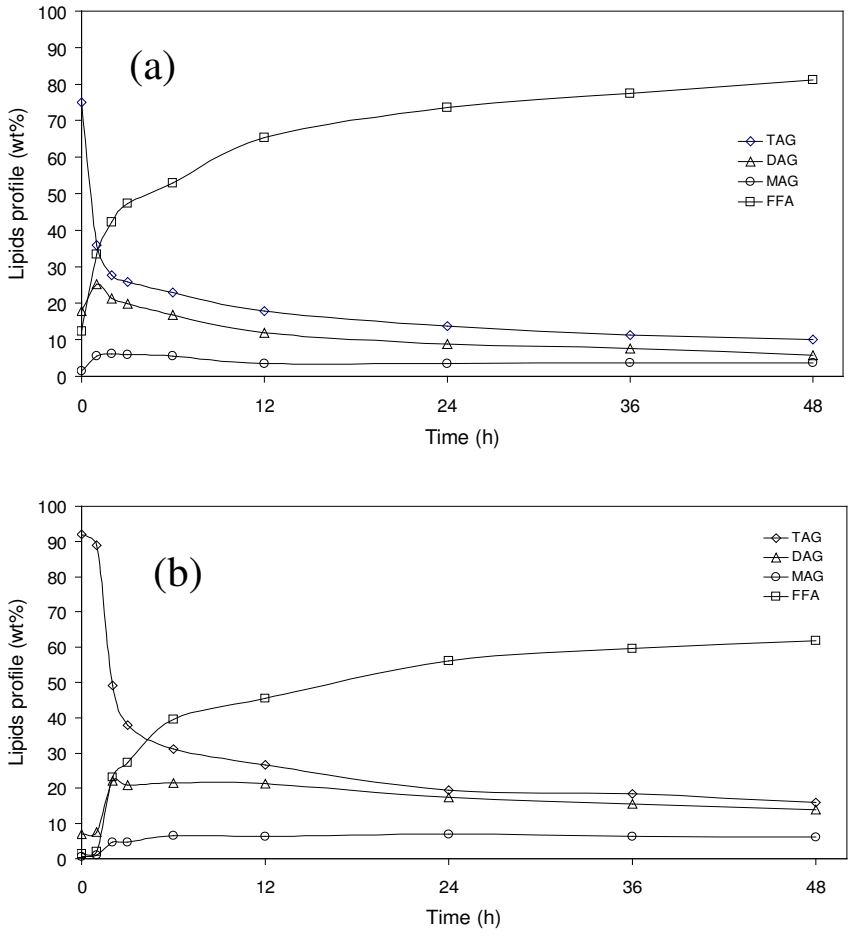
Fatty acids	**Crude oil**	**Refined oil**
Myristic acid, (C 14:0)	6.02	4.02
Palmitic acid, (C 16:0)	26.78	20.78
Palmitoleic acid, (C 16:1)	5.82	5.76
Stearic acid, (C 18:0)	5.85	6.58
Oleic acid, (C 18:1)	9.73	10.73
Linoleic acid, (C 18:2)	1.22	1.68
Arachidonic acid, (C 20:4)	1.84	1.54
Eicosapentaenoic acid, EPA, (C 20:5)	5.42	6.42
Docosahexaenoic acid, DHA, (C 22:6)	27.18	28.18
Others	10.01	13.74

2.4.2 Optimization of PUFA-rich FFA

2.4.2.1 Hydrolysis of tuna oil by different immobilized lipases

The enzyme used to hydrolyze tuna oil for PUFA-rich free fatty acids was selected. Figure 4 showed the hydrolytic products from tuna oil by immobilized enzymes from

lipase PS, AK and D. The results showed that the content of TAG, DAG, MAG was decreased after 3 h while content of FFA was increased. Immobilized Lipase D and Lipase PS provided the highest content of FFA above 80 wt% at 48 h. After 6 h of hydrolysis by Lipase D, around 60% of FFA was achieved with 7.76% of EPA and 33.74% of DHA were hydrolyzed as FFA in the mixture. The highest content of PUFA in FFA mixture of hydrolyzed tuna oil were 8.31% EPA and 34.74% DHA at 24 h of hydrolysis, while the immobilized lipase PS gave 7.51% EPA and 22.53% DHA at 24 h (Figure 5). From these results, the specificity of lipase D and PS are flavored to hydrolyze the group of PUFA (EPA and DHA) in the tuna oil mixture than lipase AK which are non specific enzyme (Suzuki *et al*., 1988; Shimada *et al.,* 1997b). This hydrolyzed tuna oil was subjected to the next experiment for extraction of FFA.



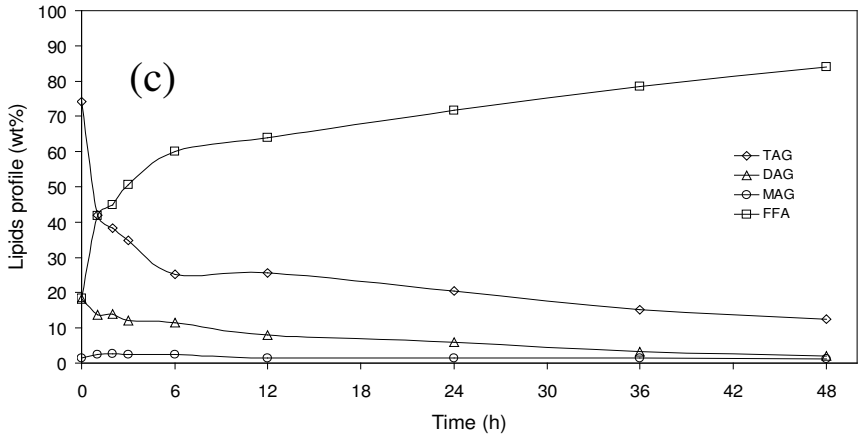
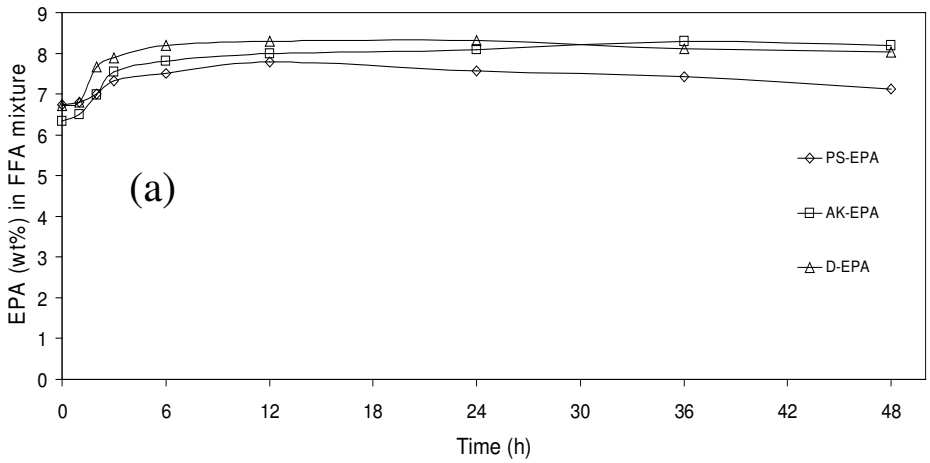


Figure 4. Profiles of glyceride species during hydrolysis of tuna oil by different lipases. (a) Lipase PS; (b) Lipase AK; (c) Lipase D. Reaction mixture: fish oil 15 g, water 15 g immobilized lipase 100U/g mixture. The reaction was carried out by stirring at 500 rpm and 40 °C.



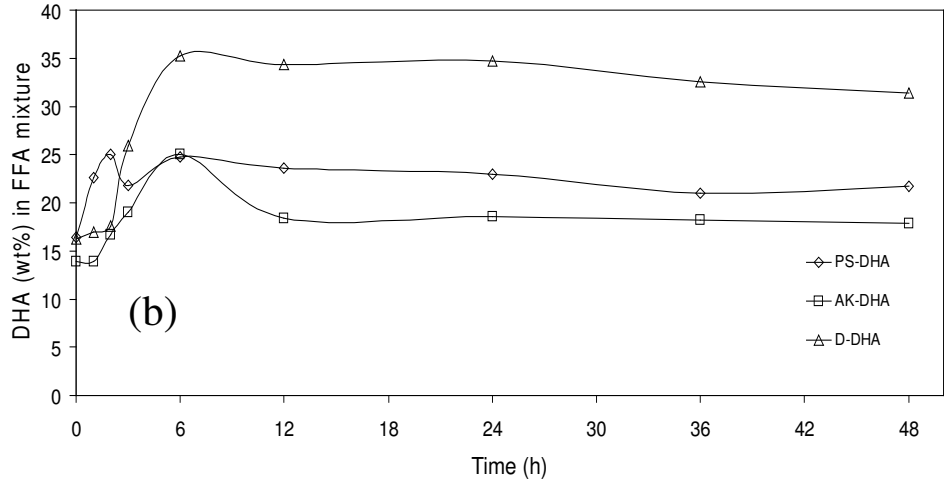


Figure 5. The contents of (a) EPA and (b) DHA during hydrolysis of tuna oil by different immobilized lipases. Reaction mixture: fish oil 15 g, water 15 g immobilized lipase 100U/g mixture. The reaction was carried out by stirring at 500 rpm and 40 °C.

2.4.2.2 Separation of FFA from the reaction mixture

The FFA mixture was separated from hydrolyzed tuna oil according to the method of Senanayake and Shahidi (1999) and Wanasundara and Shahidi (1999). The results showed that hydrolyzed FFA (82% from the hydrolyzed tuna oil) contained 8.31 % of EPA and 34.74% of DHA (Table 14). This FFA mixture was subjected to the next experiment for enrichment of EPA and DHA.

2.4.2.3 Enrichment of PUFA by selective esterification

The FFA mixture was selective esterified with octanol by different immobilized lipases to enriched PUFA. The results showed that immobilized Lipase AK (100U/g mixture, 50 °C for 24 h) gave the highest content of free fatty acids (63.8 wt%) in reaction mixture (Figure 6). From Figure 7 showed the highest contents of PUFA (10.31 % EPA and 50.74% DHA) in PUFA concentrate when immobilized lipase AK was used comparing to the other immobilized lipases. The studied of Rakshit *et al.* (2000) showed that shot-chain alcohol had little selectivity towards unsaturated fatty acid with low levels of esterification, while long-chain alcohol enhanced the esterification with a considerable amount of selectivity. Shimada *et al.* (1997a; 1997b) observed a similar phenomenon with lipase enzyme from *Rhizopus delemar* on tuna oil with recovery of 71%. However, the enzyme they used was not immobilized and the temperature of reaction is only 30 °C.

2.4.2.4 Enrichment of PUFA by urea complexation

The fatty acid compositions of refined tuna oil, hydrolyzed FFA and the PUFA concentrates obtained by selective esterification and urea complexation are presented in Table 14. Fractionation results show a total reduction in saturated FFA content (C 14:0, C 16:0 and C 18:0) and also a large reduction in monounsaturated FFA (C 18:1) but remarkable increase in PUFA (EPA and DHA). The urea complexation of FFA mixture provided PUFA concentrate of 50.40 wt%. The contents of EPA and DHA in the PUFA concentrate obtained from urea complexation were 19.50 and 56.21 %, respectively. Ratnayake *et al.* (1989) demonstrated pilot-scale (20 kg) urea complexation for concentrating PUFA compared with the other methods for producing PUFA concentrates, urea fractionation allows handling of large quantities of materials in simple equipment. Since the process requires only limited use of less toxic organic solvents such as ethanol, it is environmentally friendly. It is also cost-effective because urea is relatively inexpensive. Moreover, Klinkesorn *et al.* (2004) also demonstrated that urea complexation could eliminate C 16:0 and C 18:1 while EPA and DHA were enriched. Thus, EPA and DHA in tuna oil could be enriched by urea complexation.

2.5 Conclusion

Synthesis of polyunsaturated fatty acid-rich fish oil by two-step enzymatic method consists of hydrolysis of tuna oil and selective esterification of resulting FFA. The results showed that Lipase D hydrolyzed the ester of EPA and DHA as strongly as those of the other constituent fatty acids and was suitable for the first-step hydrolysis. Lipase AK was suitable for the second-step esterification with octanol because it acted only weakly on PUFA. Combination of enzymatic hydrolysis with urea complexation has the advantage to obtain highly enriched PUFA from FFA mixture by crystallization of other fatty acids at low temperature.

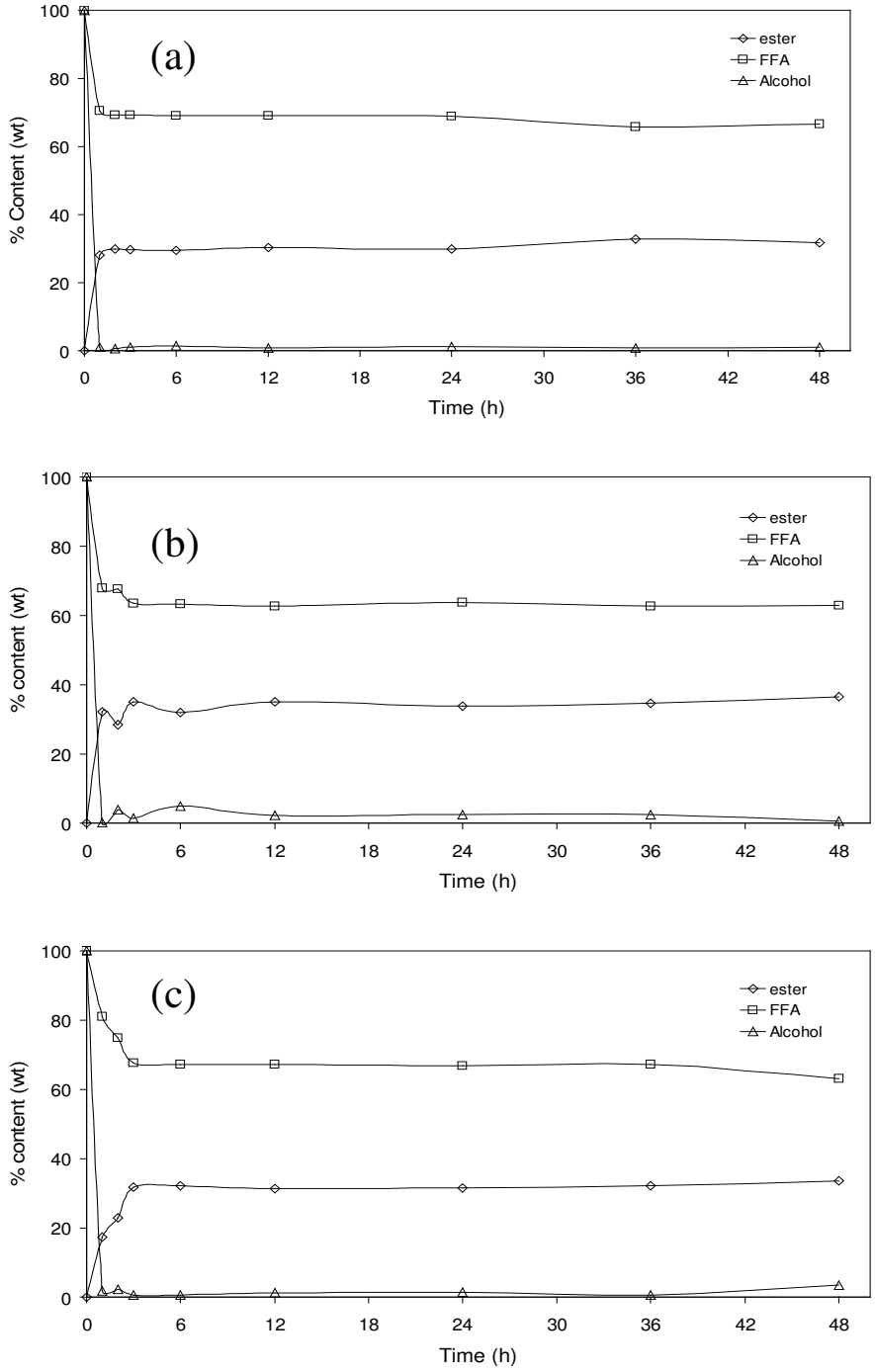


Figure 6. Selective esterification of free fatty acids with octanol by different immobilized lipases. (a) Lipase PS; (b) Lipase D; (c) Lipase AK. Reaction mixture: hydrolyzed FFA obtained from hydrolyzed tuna oil: octanol (1:3 mol/mol) (4 g), water (1 g) and immobilized lipase (100 U/g. mixture), reaction condition stirring at 500 rpm and at 30 °C for 48 h.

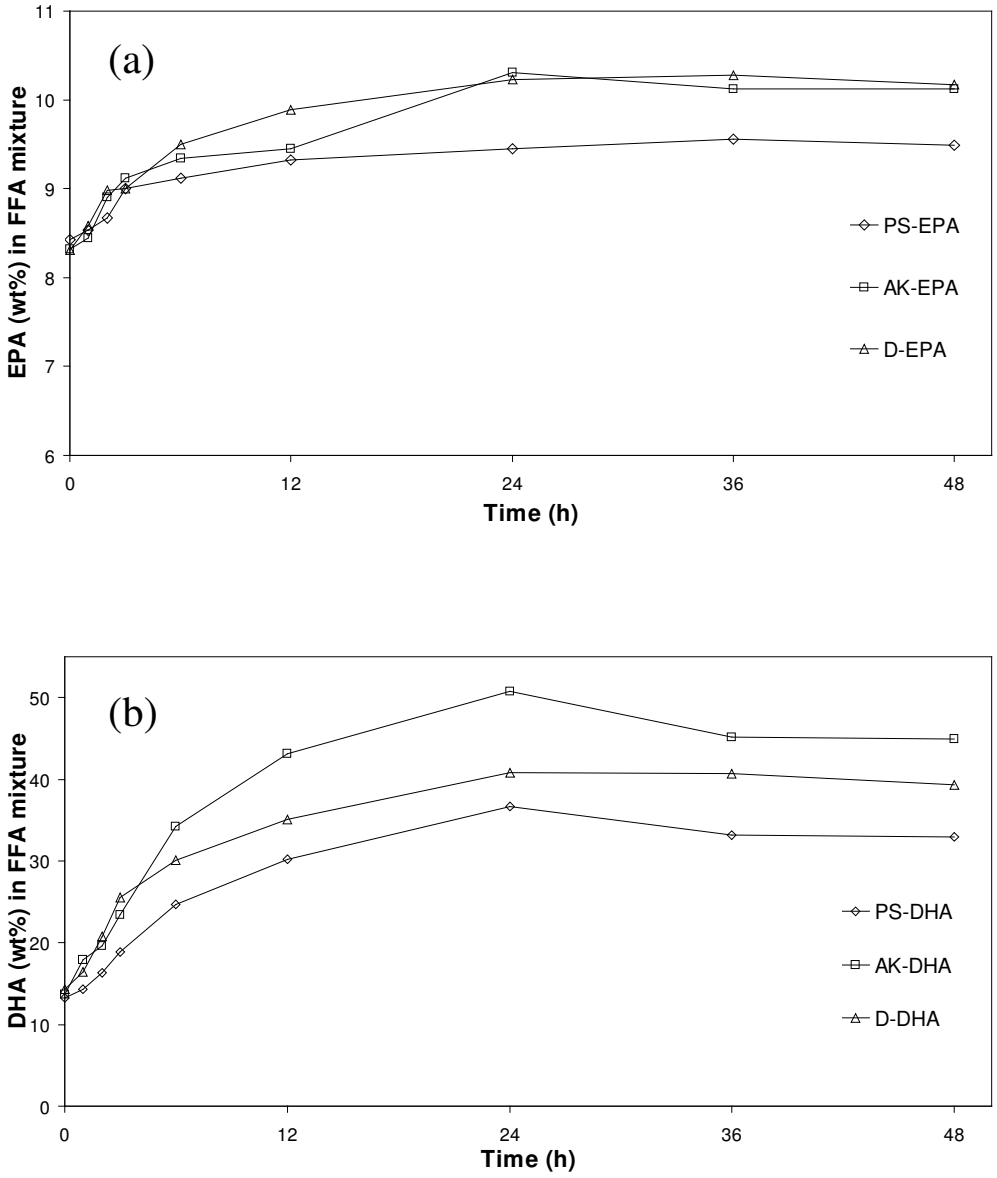


Figure 7. The contents of (a) EPA and (b) DHA by selective esterification of hydrolyzed FFA mixture with octanol by different immobilized lipases. Reaction mixture: hydrolyzed FFA obtained from hydrolyzed tuna oil: octanol (1:3 mol/mol) (4 g), water (1 g), immobilized lipase (100 U/g. mixture) and stirring at 500 rpm at 30 $^{\circ}$C for 48 h.

Table 14. Fatty acid compositions of hydrolyzed tuna oil and FFA mixture after selective esterification and urea complexation

Fatty acid	Hydrolyzed	FFA mixture*	
(% peak area)	tuna oil	after selective esterification**	after urea complexation
C14:0	3.09	1.55	-***
C16:0	20.45	2.25	-
C16:1	5.78	3.28	4.50
C18:0	4.12	1.80	-
C18:1	5.34	2.65	2.48
C18:2	1.90	3.55	-
C20:4	1.89	1.32	-
C20:5	8.31	10.31	19.50
C22:6	34.74	50.74	56.21
Others	15.23	15.67	17.35
EPA+DHA	43.05	61.05	75.71

* Extraction of FFA was carried out according the method of Senanayake and Shahidi (1999) and Wanasundara and Shahidi (1999).

** Selective esterification of FFA mixture with octanol by immobilized lipases AK. The reaction was carried out at 30 °C for 48 h, hydrolyzed FFA:octanol (1:3 mol/mol) 4 g, water 1 g and immobilized lipase AK (100 U/g mixture)

*** Not detected