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Enrichment of polyunsaturated fatty acids from tuna oil using immobilized *Pseudomonas fluorescens* lipase

S. K. Rakshit, R. Vasuhi, Y. Kosugi

Abstract Immobilized *Pseudomonas fluorescens* lipase enzyme was used to enrich the important polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) from tuna oil. Hydrolysis, esterification, and transesterification reactions were studied in detail to find out the fractionation pattern of DHA and EPA during these processes due to preferential selectivity for or against these PUFA. Hydrolysis with *P. fluorescens* biotype I lipase with stoichiometric amount of water content gave more than 80% of DHA and EPA in the free fatty acid (FFA) form after around 60% of hydrolysis. After some preferential specificity during the early stages of hydrolysis, *P. fluorescens* lipase exhibits nonselective characteristics on extended hydrolysis. Esterification of FFA extracted from the completely hydrolyzed mixture of tuna oil was found to be better with long chain fatty alcohol like octanol which lead to good enrichment (44.5% for DHA and 11.3% for EPA) and yields of the PUFA in the FFA form. Transesterification (ethanolysis) with immobilized *P. fluorescens* lipase enzyme resulted in good enrichment and recovery of DHA and EPA in the glyceride mixture. After around 60% of ester synthesis, 74% of (DHA + EPA) enrichment was achieved with yields of more than 90% in the glyceride mixture.

1 Introduction

The *n*-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to be of major importance in the prevention or treatment of a range of human diseases or disorders. However, clinical and nutritional studies are retarded by the unavailability of commercial preparations of purified DHA and EPA [1]. Marine oils are readily available major sources of *n*-3 fatty acids. The recognition that these fatty acids present in fish oil play an important role in human health and nutrition has led to much research into methods of extracting and concentrating these materials from marine oils. There are a number of physical fractionation methods like urea inclusion [2, 3], super critical fluid extraction [4] and combinations of such methods [5] which have been used to purify the DHA and EPA from fish oils. Exposure of the fish oil to high temperatures, solvents and adverse conditions during these processes may produce thermal degradation and oxidation of PUFA [6].

Another approach is an enzymatic process making use of lipase enzymes. Lipases have distinct advantages compared to classical chemical catalysts. They function under mild reaction conditions, there is little formation of side products and little or no thermal degradation of products. Several methods have been investigated to enrich PUFA in different forms such as glycerides, free fatty acids (FFA) and their esters by enzymatic treatment [7–9]. Selective hydrolysis with acyl specific *G. candidum* lipase enzyme gave recovery of 85% of DHA and EPA in glyceride mixture [1]. Selective esterification of evening primrose oil by lipozyme gave 7- to 9-fold enrichment in the glyceride form of these PUFA [10]. In our laboratory attempts were made to enrich PUFA from tuna oil, which has the largest concentration of PUFA as compared to other fish oils, through hydrolysis, transesterification, and esterification reactions with the immobilized *Pseudomonas fluorescens* lipase enzyme as catalyst.

2 Materials and methods

2.1 Materials

Immobilized lipase enzyme, Enzylon PF, was obtained from Rakto Kasie Co. Ltd. (Ootsu, Japan). More than 2500 units of lipase enzyme obtained from *Pseudomonas fluorescens* was adsorbed per gram of Dowex MWA-1. Tuna oil was obtained from the T.C. Union Agrotech Company Limited, Bangkok, Thailand.

2.2 Assay methods

Hydrolysis of tuna oil was carried out in a 125 ml Erlenmeyer flask reactor from which all the air had been removed by sparging N_2 gas into the reacting solutions initially. The reaction mixture was then incubated in a shaking water bath at 200 rpm and a temperature of 50 °C. Hydrolysis was carried out with 20% immobilized enzyme load based on the weight of oil and stoichiometric water content. Samples were collected at appropriate intervals and the acid value of the mixture was measured by titra-

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S. K. Rakshit (✉), R. Vasuhi
Bioprocess Technology Program, Asian Institute of Technology, PO Box 4, Klong Luang, Pathumthani 12120, Thailand

Y. Kosugi
National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, 1-1 Higashi, Tsukuba 305, Japan

tion with 0.1 N ethanolic KOH and phenolphthalein indicator. Free fatty acids (FFS) was extracted and the methyl esters were analyzed using a Shimadzu gas chromatograph with a chromosorb column at 200 °C.

Esterification of tuna fish oil FFA was carried out with different fatty alcohols, namely, ethanol, propanol, octanol and glycerol. Tuna-FFA was extracted using from the tuna oil sample hydrolyzed (95%) by Enzylon under hydrolytic conditions. Five grams of this FFA was incubated with 10% alcohol (weight based on FFA) and 20% (by weight) enzyme load for 24 h. Reaction was carried out at 50 °C in the shaking water bath at 200 rpm. Acid value of the reaction mixture was measure before and after the reaction. FFA was extracted from the reaction mixtures and their methyl esters composition were analyzed for DHA and EPA content.

Transesterification was carried out with ethanol and fish oil. Reaction mixtures containing oil, stoichiometric ethanol and 10% immobilized enzyme load based on the weight of oil was incubated in a 125 ml Erlenmeyer flask that was sparged with nitrogen and sealed. The course of reaction was followed by estimating the amount of ester synthesis and the hydrolytic side reaction was followed by measuring the amount of free fatty acid released. Reaction products were separated by thin layer chromatography with the solvent system of hexane:diethyl ether:acetic acid (90:10:1 vol/vol). The spots were visualized by spraying saturated $CuSO_4$:H_3PO_4 (50:50 v/v) and followed by drying in the oven at 170 °C for 5 min. Esters were scraped from the plate and extracted with hexane and analyzed by gas chromatography without further purification. FFA was extracted with hexane with hexane and their methylesters were analyzed by gas chromatography.

3 Results and discussion

3.1 Hydrolysis of tuna oil by lipase enzyme from *Pseudomonas fluorescens*

The hydrolysis of tuna oil by lipase enzyme Enzylon PF obtained from *P. fluorescens* was followed by measuring the acid value of the resultant mixture and the hydrolysis ratio determined (Fig. 1). The quantities of the polyunsaturated fatty acids DHA and EPA were also monitored as the reaction proceeds with the help of gas chromatograph measurements and thin layer chromatography (Table 1). This was done in order to see if there was any specificity for or against these acids during the enzymatic hydrolysis reaction. According to the results, DHA and EPA was obtained in measurable quantities after 20% of hydrolysis was achieved. Around 50% hydrolysis was achieved in 24 h and 50% (wt.%) of the DHA and 40% (wt.%) of EPA were released as free fatty acid form at this point. After 48 h of hydrolysis, 80% of DHA and 75% of EPA were hydrolyzed as FFA with the concentration of 24.5 and 6.7% respectively. The results indicate that after the first 6 h of hydrolysis, concentration of DHA and EPA remained nearly the same in both the FFA and glyceride fractions. However, the weight percentage of these fatty acids increased in the FFA and decreased in glyceride fraction throughout the hydrolysis process.

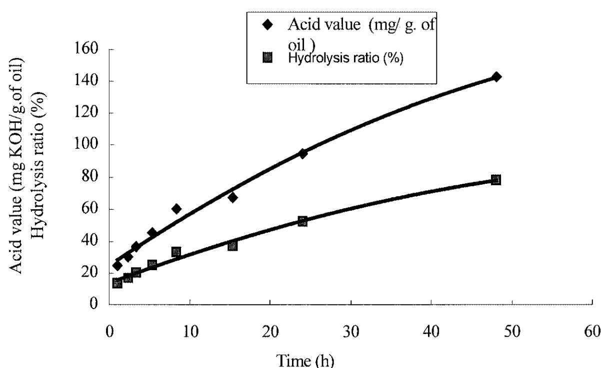

Fig. 1. Hydrolysis pattern of tuna fish oil by *Pseudomonas fluorescens* lipase

These results indicate that the enzyme used in this experiment showed minor preferential selectivity (DHA and EPA not hydrolysed) only during the first few hours of hydrolysis. Later in the hydrolysis, the enzyme does not show any selectivity and hydrolysed all the fatty acids in the same manner regardless of their position or the acyl group.

Heterogeneous systems like hydrolysis of oil, have several additional parameters like oil water ratio, stirring method, presence of surfactants and presence of organic solvents, which could affect the reaction mechanism. Hydrolysis ratio depends on these parameters and it is difficult to draw conclusions on the specificity of the lipase enzyme easily. It has been reported that *n*-3 PUFA, DHA, is highly concentrated in the position two of triglyceride, which may contribute to enzymatic discrimination [11]. The enzyme used in this study might show some positional specific characteristics at the beginning of hydrolysis. But with the progress of the hydrolysis it seems not to discriminate and exhibits a non-specific hydrolytic nature.

It has been reported that Enzylon PF hydrolysed sardine oil with slight preferential specificity for oleic and palmitic acid [12]. They observed the same non-specific characteristics of this enzyme in the higher hydrolysis ratios. At higher hydrolysis ratios this enzyme could resemble chemical catalyst, which could catalyze the oil randomly. The enzyme, however, has a higher reaction rate than the chemical catalyst and functions well under the mild reaction conditions used.

3.2 Enrichment of DHA and EPA in FFA by selective esterification

Specificity of this enzyme towards PUFA during esterification to enrich DHA and EPA in FFA form was studied. The extent of esterification and enrichment of DHA and EPA with different alcohols were measured after 24 h of reaction of the FFA acids obtained by enzymatic hydrolysis and fatty alcohols of different molecular weights (Table 2). With short chain alcohols, around 30% esterification was achieved with DHA content in the FFA portion of 30% and a yield of 85%. EPA does not show any enrichment. With glycerol, 41% esterification was achieved

Table 1. Percentage enrichment and yield of DHA and EPA in the hydrolysis products

Parameter	Form	Time (h)							Saponified oil
		2.25	3.25	5.25	8.25	15.25	24	48	
Concentration of DHA (% area)[a]	FFA	4.3	8.4	18.3	19.3	19.7	20.7	24.5	24.56
	Glyceride	28.6	28.7	26.5	27.2	27.4	28.8	23	
Concentration of EPA (% area)[a]	FFA	–	2.2	4.1	3.96	4.2	5.2	6.7	7.2
	Glyceride	–	6.5	8.2	8.8	8.9	9.1	9	
Weight % of DHA[b]	FFA	2.4	6.97	18.8	26.3	30.1	44.4	80.1	100
	Glyceride	97.1	93	80.6	73.6	69.1	55.4	19.2	
Weight % of EPA[b]	FFA	–	6.2	14.4	18.4	21.8	38.2	74	100
	Glyceride	100	92.6	85.1	81.3	77.2	59.2	25.6	
DHA/EPA ratio	FFA		3.8	4.1	4.8	4.6	4	3.8	

[a] % Fraction of DHA (or EPA) in chemical form to the total acid in that form
[b] % Fraction of the total DHA/EPA in each form

Table 2. Effect of different alcohol on esterification reaction of lipase

Alcohol	Esterification (%)	Concentration % in FFA		Yield (%wt.)[a]	
		DHA	EPA	DHA	EPA
Ethanol	30.2	30	7.9	85.5	76.7
Propanol	35.6	34.2	8.2	89.9	74.9
Octanol	55.3	44.5	11.3	81.5	70.6
Glycerol	41.4	31.2	7.2	74.4	60.2

[a] Yield % is the weight of DHA (or EPA) in FFA after the 24 h reaction to that of the weight of DHA (or EPA) in FFA before the reaction

but it subjected to high loss (by esterification) in both DHA and EPA. Long chain alcohol, octanol gave 55% esterification with considerable amount of enrichment in DHA (44.5%) and EPA (11.3%) with yield of 80% and 70% respectively after 24 h of reaction. From these results, it could be concluded that short chain alcohol shows little selectivity towards unsaturated fatty acids with low levels of esterification, while long chain alcohol enhance the esterification with a considerable amount of selectivity. Shimada et al. (1997) observed a similar phenomenon with lipase enzyme from *Rhizopus delemar* on tuna oil, but had obtained a purity of 89% with a recovery of 71%. However, the enzyme they used was not immobilized and the temperature of reaction was only 30 °C.

3.3 Transesterification of tuna oil using lipase from *Pseudomonas fluorescens*

Transesterification (alcoholysis) experiments indicate that *P. fluorescens* lipase is highly active in this reaction also. These experiments carried out under water deficient conditions with stoichiometric amounts of ethanol indicate the good tolerance of enzyme for these conditions. The enzyme also showed much lower activity towards unsaturated fatty acids (DHA, EPA) and higher activity towards saturated and mono-saturated fatty acids under these conditions (Table 3 and Fig. 2).

Weight percentages of esters and FFA were monitored as the reaction progressed. Area percentage of each lipid

Table 3. Enrichment and yield of DHA and EPA during transesterification of tuna oil using *Pseudomonas fluorescens* enzyme

Parameter	Form	Time (h)					Saponification
		4	9	15	24	48	
Concentration of DHA (%)[a]	Esters	–	1.4	1.9	4.8	16.7	
	FFA	7.9	5.7	5.5	5.1	10.2	24.6
	Glycerides	24.56	39.4	47.8	57.7	49	
Concentration of EPA (%)[a]	Esters	–	0.02	0.025	0.84	2.2	
	FFA	1.08	1.2	1.1	1.14	2.72	7.2
	Glycerides	8.4	11.8	14.7	17.9	20.5	
Weight % of DHA[b]	Esters	–	1.4	3.1	9.7	40.8	
	FFA	1.4	1.84	2.54	2.6	5.00	100
	Glycerides	98.4	96.5	94.2	87.5	53.8	
Weight % of EPA[b]	Esters	–	0.069	0.11	5.83	18.33	
	FFA	0.719	1.36	1.88	1.93	4.64	100
	Glycerides	99.3	98.3	97.3	92.1	76.8	

[a] % Fraction of DHA (or EPA) in chemical form to the total acid in that form
[b] % Fraction of the total DHA/EPA in each form

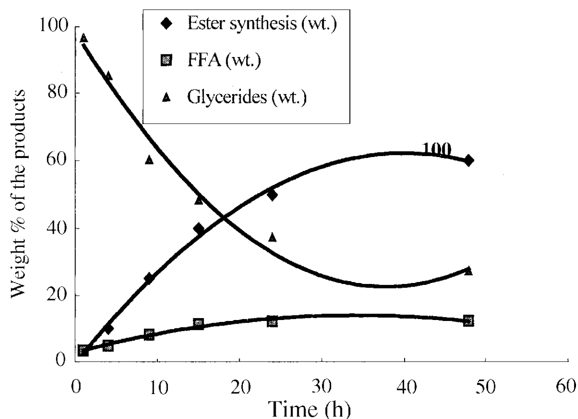

Fig. 2. Time course analysis of transesterification using *Pseudomonas fluorescens* lipase enzyme

class directly shows the enrichment of DHA and EPA along the course of reaction. Weight percentage of EPA and DHA in each class shows the yield or the recovery of DHA and EPA. As shown in Fig. 2 around 50% of esters were formed in 24 h. At this point only 9.7% of DHA and 5.8% of EPA in the form of esters (Table 3). The extent of hydrolytic side reaction, measured by the acid value of FFA present in the reaction mixture, remained below 22 (12.2 wt.%) throughout the reaction. This shows that a considerable amount of hydrolytic side reaction occurred with esterification even under these conditions. The immobilized enzyme itself seems to contain sufficient water to promote the hydrolytic side reaction. Percentage of DHA and EPA eluted in FFA form is high compared to that in esters. *Pseudomonas fluorescens* has a non-specific characteristics during hydrolysis and could explain this higher elution rate. Under these conditions, DHA and EPA were concentrated in glyceride mixture giving an enrichment of 57.7 and 17.9% respectively. This corresponds to 87.5 and 92.1% of total DHA and EPA in the glyceride mixture respectively. In the next 24 h 60% of esterification occurred of which 40% of DHA and 18.3% of EPA formed esters. Enrichment and yield in the glyceride mixture thus decreased after increasing during the first 24 h.

This result clearly shows that *P. fluorescens* lipase enzyme has acyl specificity during alcoholysis. DHA and EPA could be enriched in glycerides mixture by selective esterification of saturated and monosaturated fatty acids. Haraldsson et al. (1997) have obtained the similar results using the enzyme from a *Pseudomonas* sp. They obtained 50% EPA + DHA in the glyceride mixture with a recovery of 80% for DHA and more than 90% for EPA when ethanolysis was carried out with sardine oil at 20 °C. The rates are lower than the rates obtained in this study perhaps due to the lower PUFA in sardine oil and the lower temperature. However, those researchers did not report any substantial hydrolytic side reaction as observed in our experiments. In transesterification, a complete control of the water content is important to ensure the enzyme activity and the consequent hydrolytic side reaction, which must be kept at a minimum. This means, a compromise is required between maximum activity and the rate of hydrolysis. The enzyme used in this experiment had high moisture content. Steps should be taken to reduce the level of water content before using it in the ethanolysis reaction.

4
Conclusions

Fatty acid specificity of immobilized *P. fluorescens* lipase enzyme on hydrolysis, esterification and transesterification were different. PUFA ester bonds were hydrolyzed as easily as other acids. After 48 h of batch hydrolysis with stoichiometric water content and 20% enzyme load based on the weight of oil, 80% of DHA and 70% of EPA was found in FFA form.

Fatty acid specificity of *P. fluorescens* is highly influenced by the alcohol used as a substrate in the esterification reaction. Esterification of tuna free fatty acid with long chain fatty alcohols by *P. fluorescens* lipase enzyme gave an enrichment of PUFA in the FFA form. Esterification with octanol resulted in a yield of 80% for DHA and 70% for EPA with an enrichment of 45 and 11% respectively in the unesterified FFA.

Transesterification of tuna oil with ethanol by *P. fluorescens* lipase enzyme gave a high concentration of DHA and EPA in the glyceride mixture with high recovery. After 24 h of esterification with stoichiometric ethanol content and 20% enzyme load based on the weight of oil, recovery of more than 87% for DHA and more than 90% for EPA with an enrichment of 74% of (DHA + EPA) in glyceride mixture was obtained. *Pseudomonas fluorescens* lipase exhibits selectivity towards PUFA during ethanolysis. It esterifies saturated and monounsaturated fatty acids leaving PUFA unesterified in the glyceride mixture.

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