Immobilized Lipase – Catalyzed Fish Oil Hydrolysis in Organic Solvent

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ABSTRACT

Immobilized lipase from Mucor miehei catalyzed the hydrolysis of cod liver oil in the presence of hexane was studied. The research was conducted to observe the effect of hexane on degree of hydrolysis, glyceride profile and EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) contents. Enzymatic hydrolyses were carried out in a waterbath shaker at 50°C for 48 hours. Reaction mixtures contained of 1 g cod liver oil, hexane, Tris HCl buffer pH 7.5, and 10% (w/w oil) immobilized enzyme. Addition of various amount of hexane decreased the degree of hydrolysis and changed glyceride profile. Hexane to buffer ratio of 1:9 and 9:1 yielded 77% and 64% hydrolyses respectively. The results showed that degree of hydrolysis was affected by the oil concentration in the solvent system, not by the oil concentration in the overall system. It was observed that after hydrolysis, EPA dan DHA contents in the forms of glycreride and free fatty acid were higher in the addition of hexane than that of in the absence of hexane.

INTRODUCTION

Fish oils are important commercial source of omega-3 fatty acids. The n-3 family of polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been the focus of attention due to their role in the prevention of a number of human diseases such as cardiovascular diseases, inflammation and cancer,(Shimada, *et. al.*, 1994).The present industrial process for fats and oils hydrolysis involves high pressure and high temperature to recover 97% yield of fatty acids. This high energy process leads to polymerization and color development especially for highly polyunsaturated fatty acids.

Lipases are able to hydrolyse fats and oils with high efficiency under mild reaction conditions (Linfield, 1988, and Brandy, *et. al.*, 1988). Enzymatic hydrolysis of oils is carried out in oil-water interface. The use of lipases provide the advantages of lower energy process that simultaneously produces a colorless product. In addition, lipases are capable of catalyzing a variety of alternative enzymatic reactions, including hydrolysis and synthesis reactions of triglyceride (Macrae, 1983, Linfield, dkk., 1984, Khor, dkk., 1986, Virto, dkk., 1991, Hass, dkk., 1995, Huang dan Akoh, 1994).

Many enzymes including lipase have been known to be catalytically active in organic solvents (Mukata, *et al.*, 1987; Zak and Klibanov, 1987; Virto, *et al.*, 1991; Bilyk *et al.*, 1991; and Van Toll, *et al.*, 1995). Mukata, *et, al.*, (1987) reported that the hydrolysis level of palm oil by *Candida rogusa* lipase increased in the addition of 20% isooctane. However isooctane concentration higher than 45% tend to decrease the hydrolysis level. The more polar organic solvents such as isopropanol, diethyl ether, and chloroform seem to inhibit the enzyme activities (Virto, *et. al.*, 1991, and Fu, *et. al.*, 1995). Enzyme activities in organic solvents are influenced by many factors such as source and properties of enzymes, type and concentration of solvents and reaction conditions.

Our previous paper reported that lipase from *Mucor miehei* could hydrolyse cod liver oil at temperature ranges from 30°C to 60°C and produced more than 80% hydrolysis level (Martati, *et, al.*, 1999). This paper describe the hydrolysis of cod liver oil by immobilized lipase from *Mucor miehei* in the presence of hexane. The effect of hexane on hydrolysis level, glyceride profile and EPA and DHA contents were evaluated.

MATERIALS AND METHODS

Materials

Immobilized lipase from *Mucor miehei* (Lipozyme IM) was provided by NOVO Nordisk, Malaysia. The activity of the lipase was 117.3 µmole free fatty acid per g of enzyme per minute. Refined cod liver oil was imported from Soma Chemie Germany and stored in a dark bottle. Olive oil for enzyme assay was obtained from Sigma Co. Tris-buffer, organic solvents and other chemicals were purchased from Merck.

Hydrolysis of cod liver oil with lipase

Enzymatic hydrolyses were carried out in a 100-ml flask. Reaction mixtures contained of 1 g cod liver oil, 10 ml Tris-HCl buffer pH 7.5, 10% immobilized lipase (w/w oil) and various amounts of hexane or heptane. Hydrolyses of cod liver oil were also carried out in various hexane: buffer ratio to a final volume of 10 ml. The amounts of hexane and buffer used in each case are indicated in Table 1. Reaction mixtures were incubated at shaker waterbath at 50°C for 48 hours with stirring at 100 stroke per minute. After the reaction, the enzymes were separated and 10 ml acetone: ethanol (1:1) was added, and the acid value was measured by titrating with 0.2 N KOH. Degree of hydrolyses and gliceride profiles were determined at 1, 3, 6, 12, 24 and 48 hours. EPA and DHA contents in the form of glicerides and free fatty acids after 48 hour reaction time were also monitored in the presence and absence of hexane.

Analysis

Lipase activity was measured by tritating fatty acids liberated from olive oil with 0.1 N NaOH as decribed by Khor (1986). The reaction was carried out at 37°C for 30 minutes with stirring at 100 strokes per minute.

Glyceride profiles during cod liver oil hydrolyses were analysed using Thin Layer Chromatography on TLC plates with Silica gel GF 254 as a stationary phase (Mukata, *et al.*, 1987). Plate was developed in hexane: diethyl ether: acetic acid (80:20:1). The bands were visualized under ultraviolet light after spraying with 50% H_2SO_4. Kuantitatif analysis was done using TLC Scanner Camag II. Each compund obtained was presented as ratio of compound area to total area of all compounds.

For fatty acid analysis, sample was injected into Gas Chromatography analytical system in the form of methyl ester (Mukata, *et. al.*, 1987). Area of free fatty acid and non free fatty acids (mono, di, and triglycerides) were marked, separated from silica gel, and disolved in diethyl ether. Diethyl ether was evaporated, added with hexane and methylated by 2 N methanolic KOH and analysed using gas chromatography. The gas chromatography was an HP 5890 series II, equipped with a HP-5 capillary column 30 m x 0.32 mm (cross linked 5% phenyl methyl sillicone), and FID detector. The injector and detector temperatures were 270°C and 260°C respectively. Helium was the carrier and the gas flow rate was 10 ml/min.

EPA and DHA contents in the form of free fatty acids and glycerides were presented as percent ratio of EPA and DHA after to before hydrolysis.

RESULTS AND DISCUSSION

Effect of organic solvents to degree of hydrolysis of cod liver oil

Fish oil is soluble in hexane and heptane in the upper layer meanwhile aqueous buffer was in the bottom layer. Immobilized lipase was located in between aqueous and organic solvent phases.

The effect of hexane and heptane on the enzymatic hydrolysis of cod liver oil is presented in Figure 1 and 2. The result shows that hydrolysis level markedly increased during the time of reaction up to 12 hours and continuously increase in the following time. The larger the volume of hexane or heptane added to the system the lower the rate of hydrolysis. However, after 48 hours, the hydrolysis levels were not significantly different among various volume of hexane and heptane added. In this experiment, volume of buffer was set constant of 10 ml and volumes of hexane or heptane were 1, 3, 6, and 9 ml respectively. Although this condition did not affect oil : enzyme ratio, this affected the concentration of oil in the reaction mixtures, thus affected the rate of hydrolysis.

Several workers reported that addition of organic solvents could increase oil hydrolysis level by lipases such as addition of 5-10% isooctane to animal oil (Virto, *et. al.*, 1991), and 0.5 ml hexane to 1 g of palm oil (Khor, *et. al.*, 1986). Palm oil is semi solid at room temperature. Addition of hexane produces liquid palm oil in room temperature and thus improve contact between enzyme

and palm oil. In our experiment, hydrolysis was carried out at 50°C and fish oil is liquid, thus viscosity was not a problem. Our previous experiment also indicated that hexane did not responsible to the lipase inactivation. Hydrolysis of fish oil by immobilized lipase was carried out in unlimited water reaction system. Hexane is a hydrophobic solvent, and has a much lower affinity for water and is less capable of removing water from the enzyme, and therefore less likely to cause inactivation. Some workers reported that a number of lipases show substantial activity in nearly anhydrous water miscible solvents (Kirchner, *et al.*, 1985, Zaks and Klibanov, 1985,). Thus, it seems that lower oil concentration due to addition of organic solvents contributed the decrease of fish oil hydrolysis rate.

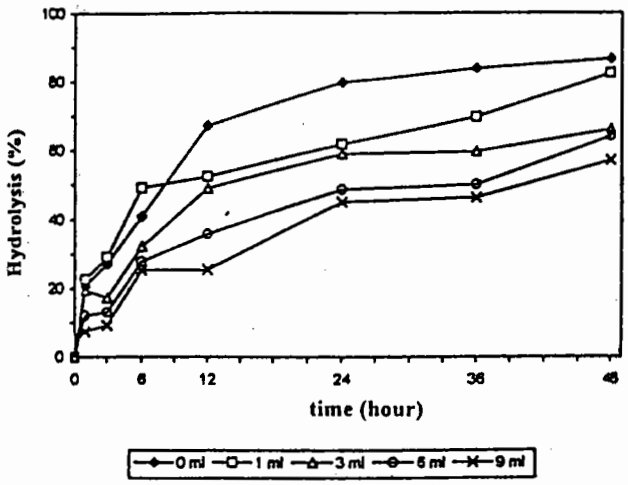


Figure 1. **Effect of hexane on hydrolysis of cod liver oil**
(1g cod liver oil, 10 ml Tris HCl buffer pH 7.5, 0.1 g Lipozyme IM, 50°C)

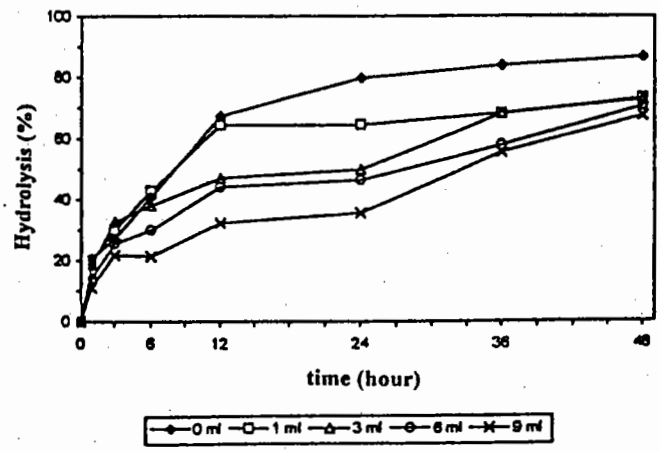


Figure 2. **Effect of heptane on hydrolysis of cod liver oil**
(1g cod liver oil, 10 ml Tris HCl buffer pH 7.5, 0.1 g Lipozyme IM, 50°C)

Glyceride profile during cod liver oil hydrolysis in the presence of hexane

Figure 3 and 4 show the glyceride profiles during fish oil hydrolysis by immobilized lipase from *Mucor miehei* in the presence 1 ml and 9 ml hexane respectively.

In reaction mixture with 1 ml hexane, the concentration of triglyceride sharply decreased in the first hour reaction, followed by gradual decreased until 48 hours. This profile corresponded to the increased in free fatty acid concentration. After 48 hours reaction concentration of MG, DG, FFA and TG were 4.9%; 7.6%; 65.5%; and 21.7% respectively. Addition of 9 ml hexane showed that the decrease in triglyceride and the increase in free fatty acid were slower and after 48 hours reaction produced 1.6% MG, 15.1% DG, 45.1% FFA and 37.9% TG. These results indicated that 9 ml of hexane markly reduced oil concentration and then decreased the rate of hydrolysis.

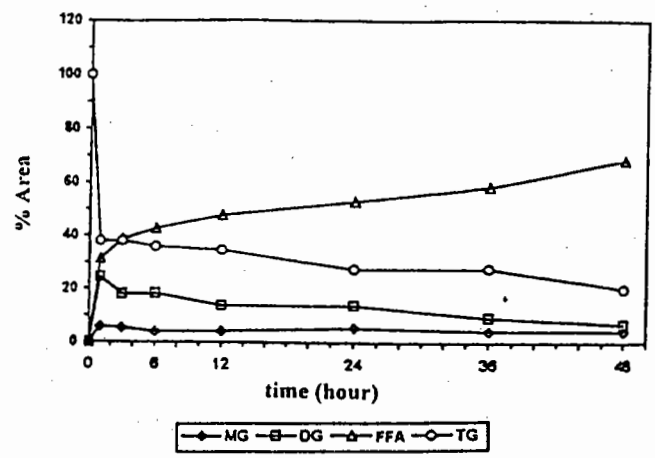


Figure 3. **Glyceride profile during fish oil hydrolysis in the presence of 1 ml hexane**
(1 g cod liver oil, 10 ml Tris HCl buffer pH 7.5; 0,1 g Lipozyme IM, 50°C)

Effect of hexane : buffer ratio on the hydrolysis of cod liver oil

In this experiment, oil hydrolyses were carried out in the various hexane:buffer ratios and constant total volume (Figure 5). Therefore concentrations of oil in the reaction system were set to 1 g/10 ml, but oil con-

centrations in the hexane phase were varied. The results show similar profile of degree of hydrolysis to results in Figure 1. The larger the volume of hexane, the lower the degree of hydrolysis. Thus larger volume of hexane tend to decrease degree of hydrolysis, either in constant or various total volume.

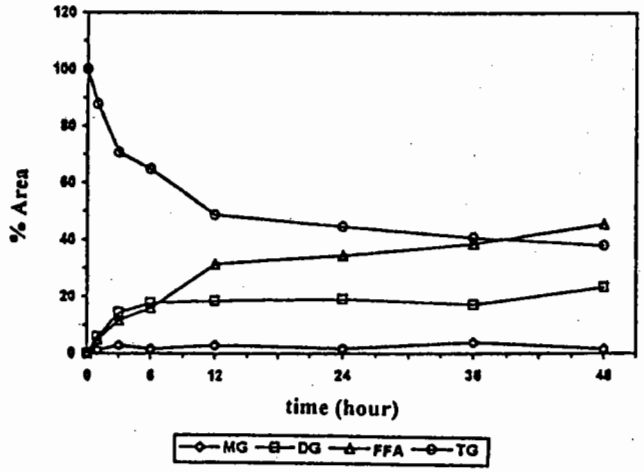


Figure 4. **Glyceride profile during fish oil hydrolysis in the presence of 9 ml hexane** (1 g cod liver oil, 10 ml Tris HCl buffer pH 7.5; 0,1 g Lipozyme IM, 50°C)

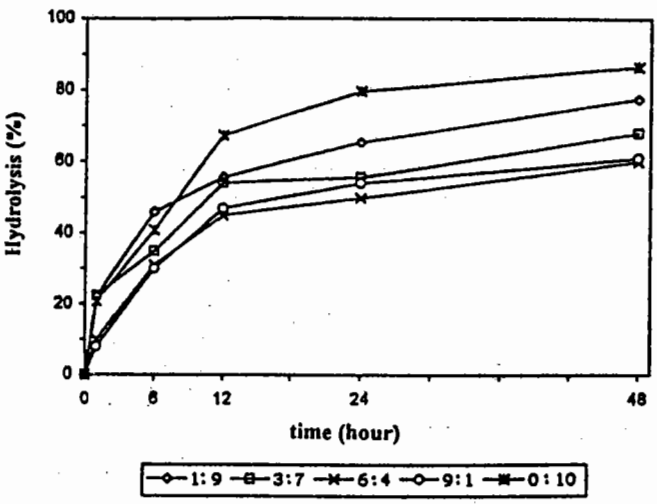


Figure 5. **Hydrolysis of cod liver oil in various hexane:buffer ratio and constant total volume.** (1 g cod liver oil; 0.1 g Lipozyme IM; total reaction volume 10 ml, suhu 50°C).

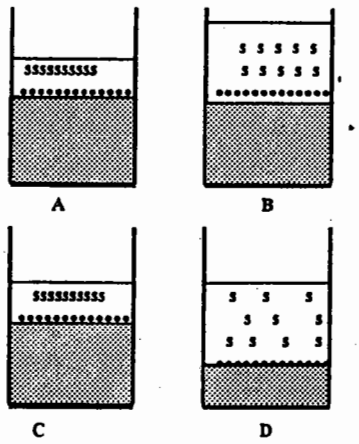
At constant buffer volume, larger volume of hexane from 1ml to 9 ml increased total reaction volume and thus significantly reduced the degree of hydrolysis from 82.5% to 57.4% (Tabel 1). However, at constant hexane volume and various buffer volume, various total reaction volume did not significantly change the degree of hydrolysis. Ratios hexane:bufer of 9:10 and 9:1 hydrolysed 57% and 64.5% respectively. In oil hydrolysis enzymatically, water and oil considerd to be substrates. Volume of water required by immobilized enzymes was reported to be ±40-50% (v/v) of water required by free enzyme (Ibrahim, *et. al.*, dkk., 1988 in Ergan, *et, al.*, 1991). Thus, 1 ml of water was sufficient for hydrolysis of 1 g cod liver oil by immobilized lipase from *Mucor miehei*. In this reaction condition, water was not a limiting factor. This results also indicated that degree of hydrolysis was influenced by concentration of oil in hexane phase not by concentration of oil in the total reaction mixtures.

Table 1. **Degree of hydrolysis of cod liver oil at various volume of hexane and buffer at 48 hours reaction**

Hexane (ml)	Buffer (ml)	Total Volume (ml)	Hydrolysis (%)
1	10	11	82,5[a]
1	9	10	77,7[a]
3	10	13	66,2[b]
3	7	10	68,0[b]
6	10	16	64,1[c]
6	4	10	60,0[c]
9	10	19	57,4[d]
9	1	10	64,1[d]

Hydrolysis of oil enzymatically involve water and oil which tend to separate to each other in the reaction system, and free enzyme is in the aqueous phase. In our experiment, oil was in the nonpolar phase of hexane, meanwhile not as free enzyme, immobilized lipase (Lypozyme IM) was in the interphase between aqueous and hexane phases (Figure 6).The rate of hydrolysis of oil enzymatically depends on contact rate among enzyme, oil and water. Water is usually not a limiting factor in hydrolysis reaction since it is available excessively. In two-phase reaction system, substrate partition between aqueous and organic solvent phases influenced substrate concentration surrounding the enzyme (Utami and Pyle.,

1998). Oil is more soluble in hexane than in water. Thus, although the absolute amount of oil in reaction mixture is constant, but the concentration of oil depends on its concentration in the hexane phase. The higher the volume of hexane, the lower the concentration of oil surrounding the enzyme (Figur 6A-B and 6C-D).



▒▒ : aqueous phase; SSS : oil in hexane; ••••• : immobilized lipase (Lipozyme IM)
A and B : fixed aqueous phase volume, varies hexane volume, fixed absolute amount of oil
C and D : fixed total volume, varies aqueous phase volume, fixed absolute of oil

Figure 6. **Model for oil hydrolysis in the presence of hexane by immobilized lipase (Lipozyme IM)**

EPA and DHA contents after fish oil hydrolysis

Table 2 shows that proporsion of free fatty acid in the hydrolysis reaction without hexane was higher than that of in the addition of 9 ml hexane since hexane reduced oil concentration. However, EPA and DHA contents in the form of free fatty acids were much lower in the absence of hexane compared to those in the addition of hexane. This could be due to degradation of polyunsaturated fatty acid including EPA and DHA. Free fatty acids of polyunsaturated fatty acid are very easy to oxidize. Addition of hexane seems to inhibit the degradation of free fatty acid.

Table 2. **EPA and DHA contents in the form of free fatty acid and glycerides after 48 hours hydrolysis of fish oil (50°C)**

Reaction Composition	Gliserida profile (%)		EPA (%)		DHA (%)	
	FFA	Glycerides	FFA	Glycerides	FFA	Glycerides
Without hexane	69,1	30.9	0,4	1.26	0,5	4,0
H : B (9:10)	45,2	54,8	4,2	58,7	4,9	79.5
H : B (9:1)	55,3	44.7	4.2	51,4	4,5	50,0

FFA : Free fatty acids
Glycerides: Mono, di and triglycerides
EPA and DHA content are presented in ratio (%) weight of EPA or DHA from the initial

CONCLUSION

Hydrolysis of cod liver oil using immobilized lipase from *Mucor miehei* in the presence of hexane decreased the degree of hydrolysis. Degree of hydrolysis was affected by oil concentration in organic solvent, and not by the concentration of oil in the whole system. Although degree of hydrolysis was higher in the enzymatic hydrolysis without hexane, EPA and DHA contents in the hydrolysate in the form of free fatty acid was considered to be low. Addition of hexane into reaction mixture seems to inhibit the degradation of free fatty acids of EPA and DHA.

ACKNOWLEDGEMENT

This work was financially supported by the Directorate General of Higher Education, Departemen of Education and Culture, Indonesia.

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