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ORIGINAL

Concentration of Docosahexaenoic Acid-containing Phospholipid through Lipozyme IM-mediated Hydrolysis

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Abstract : A selective partial hydrolyses of egg-yolk lecithin from fish-oil fed hens and squid-skin lecithin were carried out with immobilized *Rhizomucor miehei* lipase (Lipozyme IM) in *n*-hexane media in order to concentrate docosahexaenoic acid (DHA)-containing phospholipids. DHA were abundant in phospholipid fractions that had recovered from the partial hydrolysates than the corresponding substrates *i.e.* egg-yolk lecithin and squid lecithin. Thus, it was clear that DHA ester bonds were less susceptible against hydrolysis than other fatty acid ester bonds. Optimum water activity (a_w) for Lipozyme IM to concentrate DHA-containing phospholipids was $a_w=0.44$. Under this a_w , DHA showed to three fold increase compared to that of the original egg-yolk lecithin from fish-oil fed hens, after 8 h reaction at 40°C. Water activity-controlled partial hydrolysis with Lipozyme IM was borned out to be a useful method for concentrating functional phospholipids.

Key words : docosahexaenoic acid, phospholipids, concentration, water activity, Lipozyme IM

1 Introduction

Docosahexaenoic acid (DHA)-containing phospholipid plays important roles in various physiological functions¹⁾ such as the enhancement of survival of tumor-bearing mouse²⁾ and/or the differential activity of erythroleukemia cancer cells³⁾. It is also known that phospholipids and their hydrolytic products, namely lysophospholipids, show beneficial emulsifying properties^{4),5)}. Therefore, it is expected that DHA-concentrated phospholipids can exert a more health beneficial functionalities within relatively low concentration than DHA-containing simple lipids.

Lipases are often used in order to concentrate highly unsaturated fatty acids in fish oils^{6)~8)}. This is because, certain lipases show fatty acid specificity and/or positional specificity. Lipases are also capable in hydrolyzing fatty acid ester bonds in phospholipids^{9),10)}.

In this study, we report an approach for the concentration of DHA-phospholipids through selective hydrolysis of lecithins, prepared from egg-yolk of fish oil fed hens and squid-skin, mediated with immobilized lipase, the Lipozyme IM.

2 Experimental

2.1 Materials

DHA-containing lecithins prepared from egg-yolk of fish oil fed hens (DHA-egg lecithin) and squid-skin (squid lecithin) were obtained from Bizen Chemical Co., Ltd. (Okayama-ken, Japan). Phospholipid contents of DHA-egg lecithin and squid lecithin were 96.0% and 74.5%, respectively. Heptadecanoic acid methyl ester used as an internal standard was obtained from Sigma Chemical Co. (St. Louis, USA). Lipozyme IM (79.2 BIU/g), an immo-

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bilized lipase from *Rhizomucor miehei* on anion exchange resin, was a generous gift from Novo Nordisk A/S (Bagsvaerd, Denmark). All other chemicals and solvents were reagent grade.

2.2 Hydrolysis of phospholipid with Lipozyme IM

Fifty three mg of Lipozyme IM were equilibrated with saturated salt solutions in a desiccator at 25°C for 24h. Salt solutions employed were LiCl (water activity (a_w)=0.13), $MgCl_2 \cdot 6H_2O$ (a_w =0.33), K_2CO_3 (a_w =0.44), NaCl (a_w =0.75) and K_2SO_4 (a_w =0.97). To initiate the reaction, a_w -adjusted or unadjusted Lipozyme IM was added to 2mL distilled *n*-hexane solutions which contain 20mg of DHA-egg lecithin or squid lecithin. Water in *n*-hexane was not detected by Karl-Fischer titration. The reaction mixtures in screw capped vials were incubated at 40°C, 75 strokes/min under argon gas atmospheres. Reaction was terminated by removing Lipozyme IM with a 0.45 μ m PTFE filter (Gelman Japan D/N) using chloroform:methanol (1:1 vol/vol) as a solvent. The recovered reaction mixtures were applied into a silica Sep-Pak cartridge (Waters Associate Co. Ltd., Milford, USA). Free fatty acids were first removed with chloroform:methanol (10 : 1, vol/vol), then, remaining phospholipids were recovered with methanol.

2.3 Analysis

Recovered phospholipids from the hydrolysate were methylated according to the method of Christopher and Glass described by Prevot and Mordret¹¹, then analyzed by gas chromatography. Heptadecanoic acid methyl ester was used as an internal standard. A Hitachi 163 gas chromatograph equipped with a flame ionization detector (Hitachi Co. Ltd., Ibaraki-ken) and G-300 column (1.2 mm $\times$ 40 m, Chemicals Inspection and Testing Institute, Tokyo, Japan) was used. Column temperature was 195°C and the temperatures of injection and detector were 250°C. Helium was used as carrier gas at a flow rate of 10mL/min. Fatty acids were identified by retention times.

Hydrolytic degree(%) and hydrolysis resistant value (HRV) were calculated according to the following equations :

$$\text{Hydrolytic degree (\%)} = [(FA_o - FA_r) / FA_o] \times 100$$

FA_o : total fatty acid content in the phospholipid fractions of the original lecithin substrate.

FA_r : total fatty acid content in the recovered phospholipid fractions after partial hydrolysis.

$$\text{HRV (\%)} = (FA'_r / FA'_o) \times 100$$

FA'_r : individual fatty acid content in the recovered phospholipid fractions after partial hydrolysis.

FA'_o : individual fatty acid content in the phospholipid fractions of the original lecithin substrate.

Lipid composition was analyzed through thin-layer chromatography (TLC) densitometry. An aliquot of the recovered phospholipid dissolved in chloroform was applied on a silica gel TLC plate (E. Marck, Darmstadt, Germany), then developed with chloroform: methanol : 25% ammonium (65:25:5, vol/vol/vol). TLC plate were charred at 160°C for 20 min after being sprayed with 8% phosphoric acid containing 3% copper acetate¹². Plates were then subjected to densitometry. Model F-808 linearscan densitometer (Cosmo Co. Ltd. Tokyo, Japan) was used for this purpose.

3 Results and Discussion

To concentrate DHA-containing phospholipids, we examined the partial hydrolyses of lecithins with immobilized lipase (Lipozyme IM) in *n*-hexane media. DHA contents of fatty acid composition in phospholipid fractions of the original DHA-egg lecithin and squid lecithin were 11.5% and 35.6%, respectively. DHA content of fatty acid composition in the re-

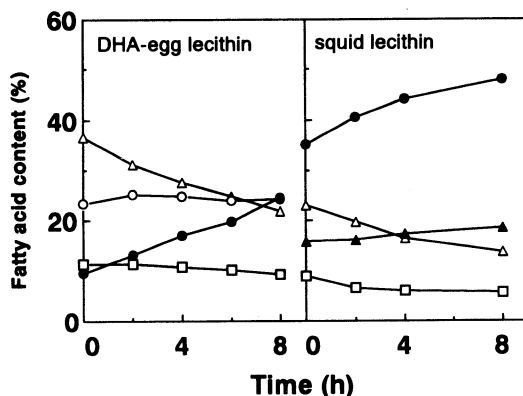


Fig. 1 Concentration of DHA through Partial Hydrolysis of DHA-containing Lecithins with Lipozyme IM.

—△— palmitic acid —□— stearic acid
 —○— oleic acid —▲— EPA
 —●— DHA

covered phospholipids separated from the resulting partial hydrolysate increased to 24.6% after 8h hydrolytic reaction at 40°C when DHA-egg lecithin was the substrate (Fig. 1). In contrast to DHA, palmitic acid content decreased and oleic acid content remained almost constant. When squid lecithin was used as substrate, DHA content of fatty acid composition in the recovered phospholipids from the partial hydrolysate increased to 48.0% after 8h reaction at 40°C (Fig. 1).

To concentrate DHA-containing phospholipids, it is necessary to dissolve the viscous DHA-containing lecithin thoroughly in order to proceed the partial hydrolytic reaction. *n*-Hexane was employed for this purpose. Hass^{9),10)} reported that Lipozyme IM-mediated hydrolyses of phospholipids in various organic solvents are drastically affected by the amount of water. Optimum water level is also dependent on solvent polarity. The effects of water levels on partial hydrolyses of DHA-containing lecithins in *n*-hexane media were therefore examined with Lipozyme IM that had adjusted to various a_w levels. The most desirable hydrolytic degree was observed when Lipozyme IM was adjusted to $a_w=0.44$. At this a_w level, hydrolytic degree was 75.5% after 8h reaction (Fig. 2). DHA content of fatty acid composition in the recovered phospholipids reached to 36.5% at this point, which is almost three

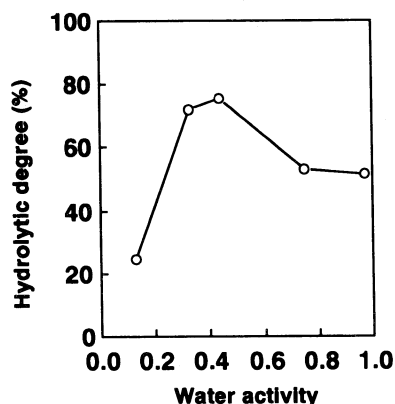


Fig. 2 Effect of Water Activity of Lipozyme IM on Partial Hydrolysis of DHA-Egg Lecithin.

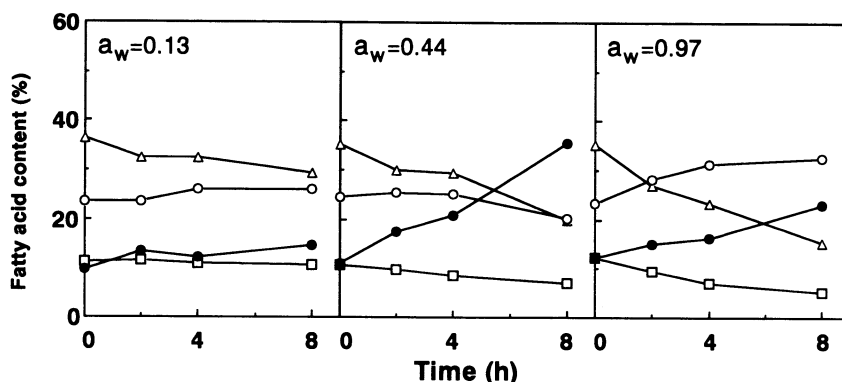


Fig. 3 Effect of Water Activity of Lipozyme IM on Concentration of DHA through Partial Hydrolysis of DHA-Egg Lecithin.

—△— palmitic acid —□— stearic acid
—○— oleic acid —●— DHA

times as much as that of the original DHA-egg lecithin (Fig. 3). At very low a_w condition ($a_w=0.13$), the hydrolytic degree was 24.6% after 8h reaction (Fig. 2, 3). DHA content increased very slowly. On the other hand, under high a_w level ($a_w=0.97$), hydrolytic degree was 51.1% after 8h reaction (Fig. 2, 3). DHA content of fatty acid composition in the recovered phospholipids was 22.9%. A little water is essential for the enzyme to be activated even in the organic media. It is assumed that water promotes the molecular flexibility of the enzyme required for catalysis¹³). But excessive amount of water often impairs the desired reaction rate^{9),10}). In this study, hydrolytic degree decreased when Lipozyme IM was adjusted to high water levels ($a_w=0.75$, $a_w=0.97$). When Lipozyme IM was adjusted to $a_w=0.44$, the highest concentration of DHA-containing phospholipids as well as the highest hydrolytic degree were obtained after 8h reaction (Fig. 2, 3). From this aspect, it is obvious that water content needs to be adjusted in a relatively lower water level in order to concentrate DHA-containing phospholipids through Lipozyme IM-mediated partial hydrolysis. Controlling water level by adjusting a_w is recommended because it is difficult to accurately adjust the slight amount of

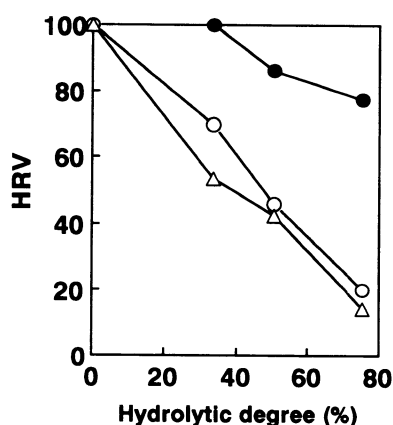


Fig. 4 Hydrolysis Resistant Values (HRV) of Fatty Acids through Partial Hydrolysis of DHA-Egg Lecithin with Lipozyme IM adjusted to $a_w=0.44$.

—△—palmitic acid —○—oleic acid
—●—DHA

water contained in the Lipozyme IM obtained from the market (a_w corresponded to 0.60). At $a_w=0.44$, the hydrolysis resistant value (HRV) of DHA was much higher than those of palmitic acid and oleic acid (Fig. 4). The HRV of DHA was still 100% when hydrolytic degree was 33.5%. DHA ester bonds were clearly less susceptible against hydrolysis than the other fatty acid ester bonds.

We also examined the concentration of DHA-containing phospholipids when squid lecithin was used as substrate. At $a_w=0.44$, DHA content of fatty acid composition in the recovered phospholipids reached to 59.7% after 8h reaction (Fig. 5). At $a_w=0.13$ and $a_w=0.97$, DHA contents of fatty acid composition in the recovered phospholipids increased to 42.0% and 55.0% after 8h reaction, respectively. DHA was concentrated effectively. HRV of DHA at $a_w=0.44$ was the highest reaching to 83.9% (Fig. 6). HRVs of palmitic acid and icosapentaenoic acids (EPA) were 17.6% and 62.1%, respectively. It has been reported that Lipozyme IM preferentially acts on fatty acid ester bonds in *sn*-1 position of phosphatidylcholine (PC)⁹⁾. DHA is mostly esterified on *sn*-2 position in DHA-egg phospholipids and in squid-skin phospholipids. Therefore, it is considered that the positional specificity of Lipozyme IM might contribute at first for concentrating DHA. Then, acyl chain specificity should also contribute, because HRV of DHA was higher than that of EPA esterified in the same posi-

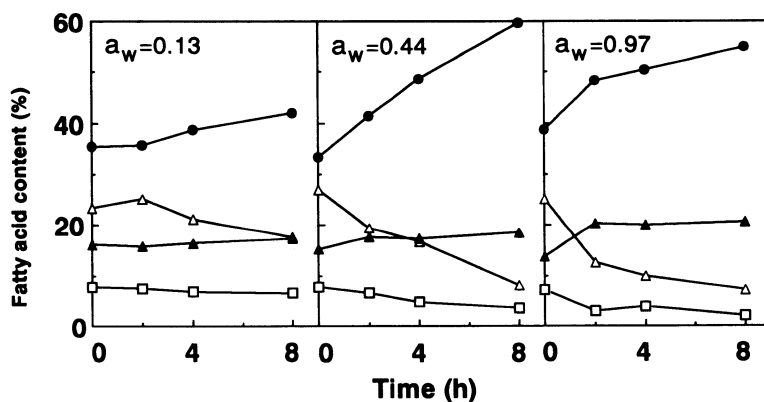


Fig. 5 Effect of Water Activity of Lipozyme IM on Concentration of DHA through Partial Hydrolysis of Squid Lecithin.

—△— palmitic acid —□— stearic acid —▲— EPA —●— DHA

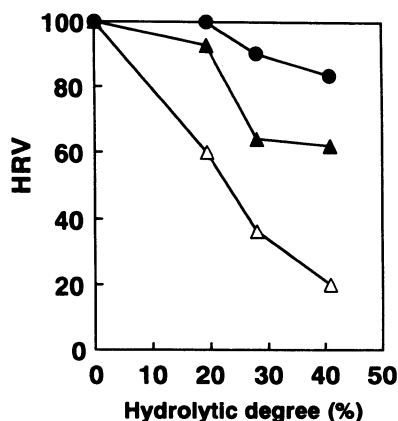


Fig. 6 Hydrolysis Resistant Values (HRV) of Fatty Acids through Partial Hydrolysis of Squid Lecithin with Lipozyme IM Adjusted to $a_w=0.44$.

—△— palmitic acid —□— stearic acid —▲— EPA —●— DHA

Table 1 Lipid Composition and Yield of Recovered Phospholipids after Partial Hydrolysis of Lecithins with Lipozyme IM Adjusted to $a_w=0.44$.

	Yield(%) ^a	PC	PE	Sph	LPC	LPE	Others ^b
Recovered phospholipid reactant from DHA-egg lecithin hydrolysate	40.3%	30.0 (71.4)	13.8 (22.7)	7.6 (2.5)	34.7 (3.4)	13.9 (0.0)	— (—)
Recovered phospholipid reactant from squid lecithin hydrolysate	69.5%	26.1 (48.7)	18.2 (18.5)	12.0 (9.0)	18.3 (0.0)	3.2 (5.5)	22.2 (18.3)

a : Yield(%)=(weight of the recovered phospholipids after partial hydrolysis/weight of the phospholipid fractions in original lecithin substrate)×100.

b : Spots on thin layer chromatograph that had visualized with 3% copper acetate solution, but not detectable with Dittmer reagent.

Lipid compositions of the phospholipid fractions in original lecithin substrates are shown in parentheses.

Abbreviations : PC, phosphatidylcholine ; PE, phosphatidylethanolamine ; Sph, sphingomyelin ; LPC, lysophosphatidylcholine ; LPE, lysophosphatidylethanolamine.

tion.

Lipid compositions of the recovered phospholipids after 8h partial hydrolytic reactions with Lipozyme IM adjusted to $a_w=0.44$ are shown in **Table 1**. Lysophosphatidylcholine(LPC) and lysophosphatidylethanolamine (LPE) were derived from phosphatidylcholine (PC) and phosphatidylethanolamine (PE), respectively through the corresponding partial hydrolyses. LPC contents were 34.7% and 18.3% in the recovered phospholipids obtained from the partial hydrolysates of DHA-egg lecithin and squid lecithin, respectively. LPE contents in the recovered phospholipids were 13.9% and 3.2%, respectively in those substrates. Yields of the recovered phospholipids obtained from the hydrolysates of the corresponding substrates were 40.3% and 69.5%, respectively after 8h reaction (**Table 1**). It is well known that the emulsifying properties of lysophospholipids are more beneficial than that of diacylphospholipids¹⁴⁾. Therefore, the emulsifying properties of the recovered phospholipids derived from DHA-egg lecithin and squid lecithin must be studied.

We concluded that a_w controlled Lipozyme IM mediated partial hydrolysis is a beneficial method to concentrate functional phospholipids.

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日本油化学会誌本号掲載 論文要旨

[報文] ジミリストイルホスファチジルコリンで調製した ヘキサデカンエマルジョンの 合一過程に関する動的解析

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ジミリストイルホスファチジルコリン (DMPC) を用いて調製したヘキサデカンエマルジョンの合一過程を調べた。予め 25℃ と 35℃ で一昼夜熟成させた DMPC/水分散系によってヘキサデカンを乳化し、これらのエマルジョンの粒子径の経時変化を 25℃, 29℃, 35℃ で測定した。これらのエマルジョンの合一過程に 1 次と 2 次の速度式を適用して合一の速度定数 (k) を求め、アレニウス式から合一の活性化エネルギー (E^*) を求めた。35℃ で調製したエマルジョンの k_{35} の値は 25℃ で調製したエマルジョンの k_{25} の値より数倍大きく、35℃ で調製したエマルジョンの E^*_{35} の値は 25℃ で調製したエマルジョンの E^*_{25} の値の約半分となることがわかった。これらの合一速度のパラメーターの相違を 25℃ と 35℃ で調製されたエマルジョンの粒子表面における DMPC の吸着状態の相違によって説明した。

(連絡者：田嶋 和夫) Vol. 46, No. 8, 857 (1997)

[報文] リポザイム IM を用いた加水分解反応による ドコサヘキサエン酸結合型リン脂質の濃縮

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ドコサヘキサエン酸 (DHA) 結合型リン脂質を濃縮するため、*Rhizomucor miehei* 由来のリパーゼを固定化した酵素 (Lipozyme IM) を用いて、 n -ヘキサン溶液中でのグリセロリン脂質の選択的部分加水分解反応について検討を行った。DHA 強化卵黄レシチンおよび、イカ皮より抽出したレシチンを部分加水分解することにより、リン脂質画分中に DHA のさらなる濃縮がみられた。特に、DHA は他の脂肪酸に比べ加水分解を受けにくいので、リン脂質画分中に最も選択的に濃縮された。Lipozyme IM の水分活性 (a_w) を 0.44 に調節した場合において DHA の濃縮が最も顕著であり、DHA 強化卵黄レシチンを 40℃ で 8 h 加水分解したところ、回収物リン脂質画分中の DHA 濃度が、基質である DHA 強化卵黄レシチンのリン脂質画分中の DHA 濃度の 3 倍以上にまで達した。

以上の結果より、水分活性を調節した Lipozyme IM を用いた部分加水分解により機能性リン脂質の効果的な濃縮が可能であることが明らかとなった。

(連絡者：高橋是太郎) Vol. 46, No. 8, 867 (1997)