Water Activity-Adjusted Enzymatic Partial Hydrolysis of Phospholipids to Concentrate Polyunsaturated Fatty Acids

Masayo Ono[a], Masashi Hosokawa[a], Yoshikazu Inoue[b], and Koretaro Takahashi[a,*]

[a]Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan, and [b]Bizen Chemical Co., Ltd., Okayama 709-07, Japan

ABSTRACT: Selective partial hydrolyses of egg yolk phospholipid and squid skin phospholipid were carried out. By keeping the water activity (a_w) of Lipozyme IM at an intermediate level, it was easy to concentrate docosahexaenoic acid (DHA). It was also possible to concentrate both DHA and arachidonic acid (AA) simultaneously to a certain level under this a_w range. However, it was impossible to concentrate AA alone when DHA was present. Though there is a limitation in concentrating AA exclusively, the proposed a_w-adjusted hydrolytic reaction is a promising way for preparing phospholipids rich in DHA.
JAOCS 74, 1415–1417 (1997).

KEY WORDS: Arachidonic acid, DHA, docosahexaenoic acid, formula, hydrolysis, lipase, Lipozyme, partial hydrolysis, phospholipid, water activity.

Preterm infants rely on formulae to obtain fatty acids essential for normal development, particularly of the visual system. Docosahexaenoic acid (DHA) has been supplemented in formulae for this purpose. However, relatively low arachidonic acid (AA) levels in formulae may be associated with poor growth in formula-fed preterm infants. Hoffman and Uauy (1) emphasized that both n-3 and n-6 polyunsaturated fatty acids (PUFA) should be provided by the formula. Yonekubo (2) also pointed out that well-balanced doses of DHA, AA, and cholesterol should be important. At the world conference on "Highly Unsaturated Fatty Acids in Nutrition and Disease Prevention," held in Barcelona last December (1996), these indications were also confirmed through many discussions.

Pertaining to the recommended levels of DHA and AA in formulae for preterm infants, several guidelines have already been proposed (Table 1).

The purpose of the present study is to design a partial hydrolytic reaction of phospholipids (PL) prepared from egg yolk, from egg yolk of fish oil-fed hens, or from squid skin, mediated by Lipozyme IM to concentrate DHA and AA so as to meet the requirement of developing an ideal formula.

*To whom correspondence should be addressed at Laboratory of Food Biochemistry, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato, Hakodate 041, Japan. E-mail: ktak@fl.hines.hokudai.ac.jp.

TABLE 1
Guidelines Proposed for Infant Nutrition

		Preterm infant		Mature infant	
Organization	Year	DHA[a]	AA[a]	DHA	AA
BNF[b]	1992	20	20	20	20
WHO/FAO[c]	1994	40	60	20	20
ISSFAL[d]	1995	35–75	60–100[e]		

[a]mg/kg weight/day. Abbreviations: DHA, docosahexaenoic acid; AA, arachidonic acid.
[b]British Nutrition Foundation.
[c]World Health Organization/Food and Agriculture Organization.
[d]International Society for the Study of Fatty Acids and Lipids.
[e]AA + docosatetraenoic acid (22:4n-6) and docosapentaenoic acid (22:5n-6).

MATERIALS AND METHODS

Materials. DHA-enriched egg yolk PL prepared from fish oil-fed hens (DHA-yolk PL), which contains 10.9% DHA and 1.5% AA, natural egg yolk PL with 3.5% DHA and 4.9% AA, and PL prepared from squid skin (squid PL), a by-product of squid processing, with 33.3% DHA and 2.7% AA, were obtained from Bizen Chemical Co., Ltd. (Okayama, Japan). Fatty acid compositions of these substrates are summarized in Table 2. The internal standard for the fatty acid analysis by gas chromatography was heptadecanoic acid methyl ester obtained from Sigma Chemical Co. (St. Louis, MO). Lipozyme IM (79.2 BIU/g), an immobilized lipase (E.C. 3.1.1.3) from *Rhizomucor miehei*, was a generous gift from Novo Nordisk A/S (Bagsvaerd, Denmark). Chemicals and solvents were reagent-grade unless stated otherwise.

Water activity (a_w) adjustment of Lipozyme IM. Prior to the hydrolytic reaction, 53 mg of Lipozyme IM was equilibrated with saturated salt solutions in a desiccator at 25°C for 24 h.

TABLE 2
Fatty Acid Composition of Egg Yolk Phospholipid (PL), DHA-Enriched Egg Yolk PL Obtained from Fish Oil-Fed Hens, and Squid Skin PL[a]

	16:0	18:0	18:1	20:4	22:6
Egg yolk PL	31.2	15.3	26.6	4.9	3.5
DHA yolk PL	35.2	10.7	24.5	1.5	10.9
Squid PL	27.0	7.8	2.7	2.7	33.3

[a]Percentage of total fatty acids. For other abbreviation see Table 1.

Salt solutions used were LiCl ($a_w = 0.13$), K_2CO_3 ($a_w = 0.44$), and K_2SO_4 ($a_w = 0.97$).

Lipozyme IM-mediated partial hydrolytic reactions of PL. Reactions were initiated by adding a_w-adjusted Lipozyme IM (53 mg each) to a reaction vial with a known weight of PL (20 mg each) dissolved in distilled *n*-hexane. Reaction mixtures were subjected to 75 strokes/min incubation in closed vials at 40°C, then quenched by passing the sample through a 0.45-μm polytetrafluoroethylene filter (Gelman, Japan D/N) with chloroform/methanol (1:1, vol/vol) to remove solid Lipozyme IM. The recovered reaction mixtures were embedded on a silica Sep-Pak cartridge (Waters Corporation, Milford, MA). Free fatty acids were first removed with chloroform/methanol (10:1, vol/vol), then the remaining PL were recovered with methanol.

Fatty acid analysis. Recovered PL from the hydrolysates were methylated according to the method of Christopher and Glass as described by Prevot and Mordret (3), 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 and a G-300 column (1.2 mm × 40 m, Chemicals Inspection and Testing Institute, Tokyo, Japan) was used.

Lipid composition analysis. An aliquot of the recovered PL, dissolved in chloroform, was applied to a silica gel thin-layer plate (E. Merck, Darmstadt, Germany), then developed with chloroform/methanol/25% ammonia (65:25:5, vol/vol/vol). Plates were then charred at 160°C for 20 min after being sprayed with 8% phosphoric acid containing 3% copper acetate (4). Quantitation was carried out by subjecting these plates to a linear scan densitometer (Model F-808; Cosmo Co. Ltd., Tokyo, Japan).

Calculation of hydrolytic degree and hydrolysis resistance value (5). Hydrolytic degree (HD) and hydrolysis resistance value (HRV), proposed by Tanaka *et al.* (5), were calculated according to the following expressions, respectively: HD = [(total fatty acid amount in substrate PL − total fatty acid amount in recovered PL fraction after partial hydrolytic reaction)/total fatty acid amount in substrate PL] × 100; HRV = (individual fatty acid amount in recovered PL fraction after partial hydrolytic reaction/individual fatty acid amount in substrate PL) × 100.

RESULTS AND DISCUSSION

Partial hydrolytic reaction of egg yolk PL under a_w-regulated conditions obviously increased the concentration of both DHA and AA in the recovered PL fraction in accordance with reaction time (Fig. 1). A relatively low intermediate a_w level, i.e., $a_w = 0.44$, seemed preferable for concentrating these PUFA. As shown in Table 3, after 24-h reaction at $a_w = 0.13$, DHA reached 15.5% from 3.5%, and AA reached 10.8% from 4.9%. However, recovery under these conditions was only 37.5%. In considering the efficiency of reaction time and recovery, it seemed beneficial to choose an intermediate a_w range, such as $a_w = 0.44$, as shown in the same table (Table 3) because the results obtained are a compromise between recovery and PUFA content within an 8-h period. Recommended HRV might be between 80 and 90 for DHA and 38 and 50 for AA, as can be derived from Table 3. HD corresponds to 73 to 75 at these HRV values.

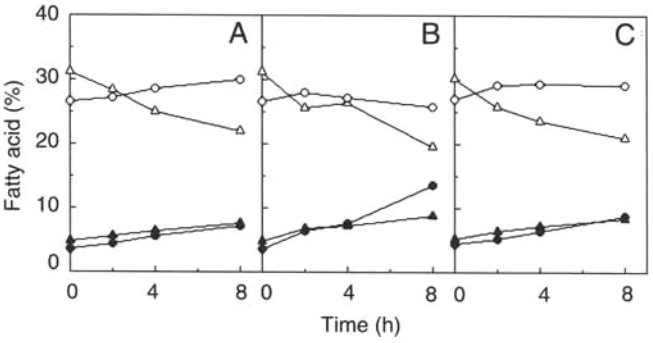

FIG. 1. Effect of water activity (a_w) of Lipozyme IM (Novo Nordisk A/S, Bagsvaerd, Denmark) on partial hydrolytic reaction of egg yolk phospholipid. (A) $a_w = 0.13$; (B) $a_w = 0.44$; (C) a_w unadjusted, i.e., $a_w = 0.60$. △, 16:0; ○, 18:1; ▲, 20:4; ●, 22:6.

Although we propose recommended conditions for concentrating both DHA and AA simultaneously, we have concluded, by analyzing the relationship between HRV and HD, that it is difficult to concentrate AA exclusively to a much higher level (Fig. 2). It seemed impossible to separate the HRV of AA from the other individual fatty acid moieties in accordance with the increase in HD, especially when DHA coexists. On the contrary, when the AA level in the substrate is low, it is feasible to concentrate DHA to a satisfactory level, as shown in Figure 3 (6). HRV of DHA obviously separates from other fatty acid moieties in accordance with an increase in HD. Figure 3 also shows that, at least up to a 20% level of HD, the DHA moiety remains intact against Lipozyme IM.

As shown in Figure 4, an intermediate a_w range seemed desirable for the enrichment reaction of DHA in PL. In fact, in fish oil-fed hen egg yolk PL, DHA increased from approximately 11 to more than 35% within 8 h at $a_w = 0.44$. Under the same conditions, DHA increased from 33 to nearly 60% in squid skin PL.

Although we can obtain highly DHA-concentrated PL from the partial hydrolysis of squid skin PL, the efficiency of DHA

TABLE 3
Recovery and Content of DHA and AA After the Lipozyme IM-Mediated Partial Hydrolytic Reaction of Egg Yolk Phospholipid

a_w	Reaction time (h)	Recovery (%)[a]	Hydrolytic degree (%)	AA (%)[b]	DHA (%)[b]
0.13	8	63.9	42.1	7.8 (83.3)	7.5 (100)
0.13	24	37.5	74.5	10.8 (50.0)	15.5 (90.0)
0.44	8	41.0	73.4	8.8 (38.5)	13.7 (80.0)
0.60[c]	8	56.4	56.1	8.0 (66.7)	7.8 (80.0)
0.97	8	60.1	50.0	8.4 (83.3)	8.0 (100)

[a]Recovery (%) = (recovered phospholipid amount after the reaction/phospholipid amount before the reaction) × 100.
[b]Parentheses are hydrolysis resistance values proposed by Tanaka *et al.* (5).
[c]Without a_w adjustment; Lipozyme IM (Novo Nordisk A/S, Bagsvaerd, Denmark).

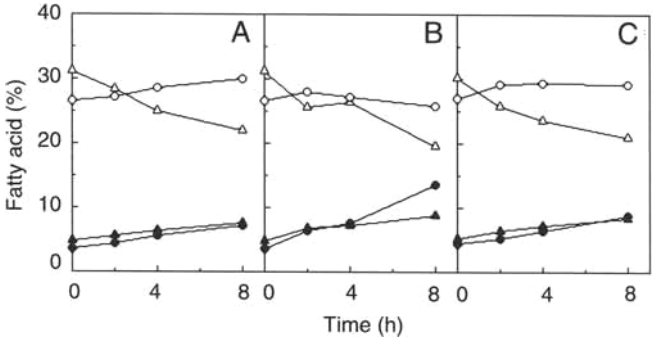

FIG. 2. Relationship between hydrolytic degree (HD) and hydrolysis resistance value (HRV) of the individual fatty acid moieties on Lipozyme IM-mediated partial hydrolytic reaction of egg yolk phospholipid at a_w = 0.44. △, 16:0; ○, 18:1; ▲, 20:4; ●, 22:6. See Figure 1 for other abbreviation and company source.

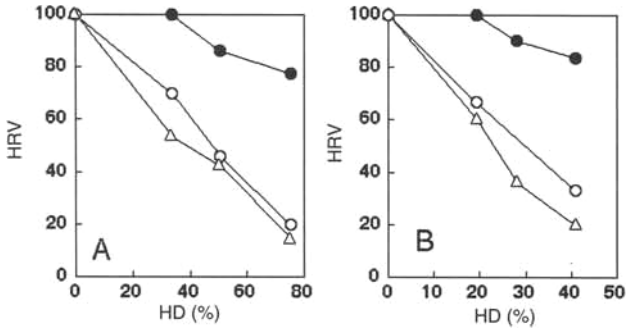

FIG. 3. Relationship between HD and HRV of the individual fatty acid moieties on Lipozyme IM-mediated partial hydrolytic reaction (A) of docosahexaenoic acid (DHA)-enriched egg yolk phospholipid, obtained from fish oil-fed hens, and (B) of squid skin phospholipid at a_w = 0.44. △, 16:0; ○, 18:1; ●, 22:6. For other abbreviations see Figures 1 and 2. For company source, see Figure 1.

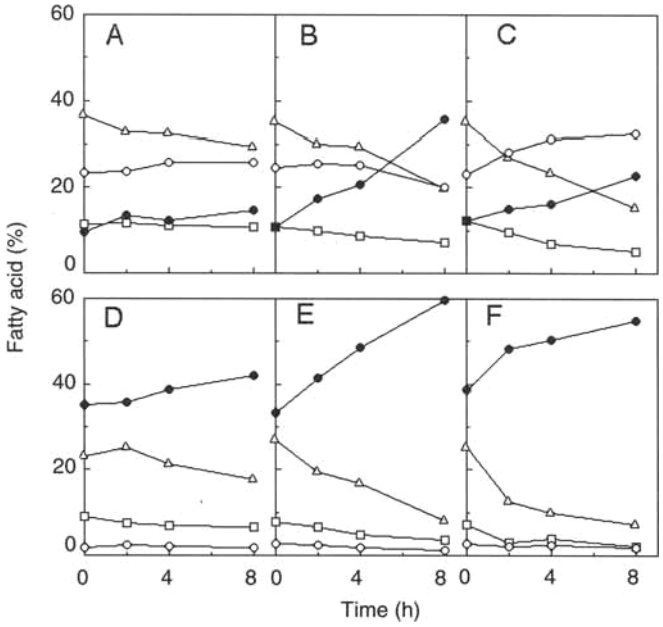

FIG. 4. Effect of a_w of Lipozyme IM on partial hydrolytic reaction of DHA-enriched egg yolk phospholipid obtained from fish oil-fed hens (A,B,C) and squid skin phospholipid (D,E,F). A,D: a_w = 0.13. B,E: a_w = 0.44. C,F: a_w = 0.97. △, 16:0; □, 18:0; ○, 18:1; ●, 22:6. For abbreviations see Figures 1 and 3. For company source, see Figure 1.

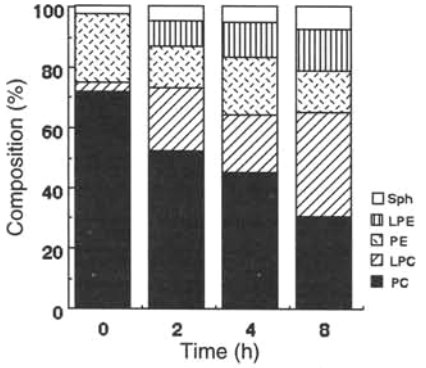

FIG. 5. Changes in lipid composition during Lipozyme IM-mediated partial hydrolytic reaction of DHA-enriched egg yolk phospholipid, obtained from fish oil-fed hens. Sph, sphingomyelin; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine. Lipozyme IM a_w was adjusted to 0.44. For other abbreviations see Figures 1 and 3. For company source, see Figure 1.

concentration seemed much higher in fish oil-fed hen egg yolk PL partial hydrolysis because there was a threefold increase in egg yolk PL and only a twofold increase in squid skin PL.

We predicted that most of the diacyl PL would change into lyso PL after the reaction, but this was not true. A considerable amount of diacyl PL still remained after 8 h of reaction, as illustrated in Figure 5. One possible explanation is that rearrangement of acyl moieties on diacyl PL occurs. Certain amounts of didocosahexaenoyl PL may have formed.

In conclusion, a_w-controlled Lipozyme IM-mediated partial hydrolytic reaction of DHA-yolk PL or PL from marine sources should be a promising technology for preparing physiologically functional PL.

ACKNOWLEDGMENT

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Hoffman, D.R., and R. Uauy, Essentiality of Dietary ω3 Fatty Acids for Premature Infants: Plasma and Red Blood Cell Fatty Acid Composition, *Lipids 27*:886–895 (1992).
2. Yonekubo, A., Saikin no shishitsu Kenkyu no Doukou [in Japanese], *New Food Industry 36(3)*:1–4 (1994).
3. Prevot, A.F., and F.X. Mordret, Utilisation des Colonnes Capillaires de Verre pour l'Analyse des Corps Gras par Chromatographie en Phase Gazeuse, *Rev. Fse. Corp. Gras. 23*:409–423 (1976).
4. Fewster, M.E., B.J. Burns, and J.F. Mead, Quantitative Densitometric TLC of Lipids Using Copper Acetate Reagent, *J. Chromatogr. 43*:120–126 (1969).
5. Tanaka, Y., J. Hirano, and T. Funada, Concentration of Docosahexaenoic Acid in Glyceride by Hydrolysis of Fish Oil with *Candida cylindracea*, *J. Am. Oil Chem. Soc. 69*:1210–1214 (1992).
6. Ono, M., M. Hosokawa, Y. Inoue, and K. Takahashi, Concentration of Docosahexaenoic Acid-Containing Phospholipid Through Lipozyme IM-Mediated Hydrolysis, *J. Jpn. Oil Chem. Soc. 46*:867–872 (1997).

[Received May 14, 1997; accepted July 24, 1997]