Application of Water Mimics on Preparation of Eicosapentaenoic and Docosahexaenoic Acids Containing Glycerolipids

Masashi Hosokawa, Koretaro Takahashi*, Noriko Miyazaki, Keiji Okamura and Mutsuo Hatano

Laboratory of Food Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate 041, Japan

ABSTRACT: To obtain enhanced incorporation of highly unsaturated fatty acids and recovery of glycerolipid products, organic solvents with high dielectric constants (water mimics) were substituted for part of the essential water for lipase activation to study their effect on acidolysis and transesterification. In acidolysis/transesterification of fish oil triglycerides and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), Lipozyme IM-60 with ethylene glycol as a water mimic enhanced the incorporation of EPA and suppressed the hydrolysis of synthesized glycerolipid. On the other hand, transesterification between soy phosphatidylcholine and EPA was enhanced by a water and propylene glycol combination. In a nonaqueous medium that contained appropriate amounts of water and organic solvents (water mimics), Lipozyme IM-60 increased transesterification of EPA into soy phosphatidylcholine. Simultaneously, the recovered glycerolipid products showed decreased hydrolysis of newly synthesized EPA- and DHA-containing glycerolipids.

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KEY WORDS: Acidolysis, docosahexaenoic acid, eicosapentaenoic acid, glycerolipids, nonaqueous solvents, phospholipids, transesterification, triglyceride, unsaturated fatty acids, water activity.

Immobilized lipase is useful for the synthesis and modification of glycerolipids that contain highly unsaturated fatty acids, which are produced in the health-related and pharmaceutical industries. The presence of water in the reaction mixture is crucial for activation of the immobilized lipase. To get a higher initial rate of incorporating fatty acids into a glycerolipid product, water activity (a_w) must be increased (1). However, under higher a_w conditions, hydrolysis is also accelerated, resulting in a lower amount of recovered product (1). An inverse relationship between the incorporation rate of polyunsaturated fatty acids and yield of glycerolipid product is observed.

*To whom correspondence should be addressed at Laboratory of Food Chemistry, Faculty of Fisheries, Hokkaido University, 3-1-1 Minato, Hakodate 041, Japan.

The present work demonstrates that enhanced incorporation of eicosapentaenoic acid (EPA) into phospholipid is observed when nonaqueous solvents are used as water mimic solvents. Reslow *et al.* (2) found that a part of the water essential for the lipase catalysis can be substituted with other polar solvents, such as formamide. Formamide has a high dielectric constant and generates hydrogen bonds like water, but it is not a substrate, and thus is thought to suppress hydrolysis. Kitaguchi and Klibanov (3) speculated that enhancement of conformational flexibility resulted in multiple hydrogen bonds with water molecules, producing activated enzymes in the organic medium. Thus, nonaqueous solvents that generate multiple hydrogen bonds can be partially substituted for water as a lipase activator.

Our work shows that ethylene glycol is a useful solvent for incorporating EPA or docosahexaenoic acid (DHA) into triglyceride (TG). With a phospholipid substrate, propylene glycol is combined with water to elicit enhanced enzymatic synthesis of EPA-containing glycerolipids.

EXPERIMENTAL PROCEDURES

Chemicals. Soy phosphatidylcholine (PC) with 95% purity was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Sardine oil was supplied by Nippon Oil Co., Ltd. (Tsukuba, Japan). Lipase (EC 3.1.1.3) from *Mucor miehei* [Lipozyme IM-60 (48.0 BIU/g) and Lipozyme IM-20 (27.5 BIU/g)] was a generous gift from Novo Nordisk Bioindustries Inc. (Bagsvaerd, Denmark). Free EPA (EPA–FFA, purity 90%), EPA ethyl ester (EPA–EE, purity 90%), free DHA (DHA–FFA, purity 88%), and DHA ethyl ester (DHA–EE, purity 88%) were kindly supplied by Nippon Chemical Feed Ltd. (Hakodate, Japan). Ethylene glycol (>95.0%, moisture 0.85%), formamide (>98.5%, moisture <0.5%), and propylene glycol (>95.0%, moisture <0.22%) were employed as water mimics and were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and solvents were reagent grade.

Acidolysis and transesterification reaction of fish oil. Water activities of substrates and Lipozyme were adjusted in

a vacuum desiccator over a salt solution or a phosphoric anhydride at 25°C. Reactions were initiated by adding 400 mg a_w-controlled Lipozyme IM-60 to a closed reaction vial containing a water-controlled mixture of 0.1 mL water mimic, 1 g fish oil, and 3 g of EPA or DHA or their ethyl esters. Reaction mixtures were subjected to magnetic stirring and incubated at 25°C for 48 h. Reactions were quenched by adding a chloroform/methanol/water (2:1:0.6 vol/vol/vol) solution. Chloroform layers were then recovered, concentrated, and subjected to preparative thin-layer chromatography (TLC) (E. Merck, Darmstadt, Germany) to isolate the EPA, DHA-rich TGs. Isolation of TG was carried out on a 0.5-mm thick silica gel 60 TLC plate (E. Merck). A developing solvent of hexane/ethyl ether (9:1, vol/vol) was followed by hexane/ethyl ether (1:1, vol/vol). Lipid composition was also analyzed through TLC at the same solvent ratio. TLC plates were charred at 150°C for 10 min after being sprayed with 8% phosphoric acid containing 3% copper acetate and were then subjected to TLC densitometry in a Model F-808 linear-scan densitometer (Cosmo Co. Ltd., Tokyo, Japan). Recovery of TG was also determined through this TLC-densitometric method. The incorporation rate of EPA or DHA to TG was defined as the EPA or DHA increase in TG fatty acid composition as determined by gas-chromatographic analysis (4).

Acidolysis and transesterification reaction of soy PC. Twenty-three mg of Lipozyme IM-60 or 40 mg of Lipozyme IM-20 were dried with phosphoric anhydride at 25°C for 24 h and placed in a screw-capped test tube. Varying amounts of water mimics, 0.5 mL of dried hexane containing 60 mg of EPA or DHA, and 10 mg of soy PC were added to the Lipozyme to initiate the reaction. The temperature of the reactions was maintained at 40°C by immersion in a reciprocal waterbath shaker (2-cm stroke at 75 rpm). Reactions were terminated by adding 5 mL of acetone/ethanol (1:1, vol/vol) and 30 mL of chloroform/methanol (1:1, vol/vol), and also by passing the reactant through a 0.45-μm pore size filter to remove the Lipozyme. Filtrates were then placed in a separatory funnel.

The recovered lipids from the chloroform layers were subjected to a silica Sep-Pak cartridge (Waters Associate Co. Ltd., Milford, MA). Free fatty acids were recovered with chloroform/methanol (10:1, vol/vol) as the eluent. PCs were isolated with methanol as the eluent. Aliquot amounts of the recovered PCs were applied to Chromarods S III (Iatron Laboratories, Inc., Tokyo, Japan) and were developed in a mixture of chloroform/methanol/water (65:25:4, vol/vol/vol). Relative amounts of the lipid classes were analyzed through TLC–flame ionization detection (FID) (Iatroscan TH-10; Iatron Laboratories).

Standard curves between weight and area (FID response) were constructed by subjecting known quantities of each lipid class to the same system. Recovery of PCs was calculated as follows:

$$\text{PC recovery (\%)} = [\text{recovered PC (mg)}/\text{applied PC (mg) before transesterification}] \times 100 \quad [1]$$

Incorporation rates (conversion) of EPA or DHA to PCs were defined as the EPA or DHA increase in PC fatty acid composition as determined by gas-chromatographic analysis (4).

RESULTS

Effect of water mimics on acidolysis and transesterification between fish oil TG and EPA and DHA acyl donors. Comparison of water mimics on EPA incorporation is illustrated in Figure 1. Among the three water mimics examined, ethylene glycol exhibited the highest incorporation rate independent of the acyl donor employed. Substrate fish oil, whose fatty acid composition included 14% EPA, increased that level to more than 70% when 90% purity EPA–FFA or EPA–EE was used as acyl donor. Formamide and propylene glycol slightly inhibited transesterification between TG and EPA–EE.

Figure 2A exhibits the effect of ethylene glycol on EPA incorporation into fish oil TG under various a_w conditions. Ethylene glycol clearly increased EPA incorporation. It increased

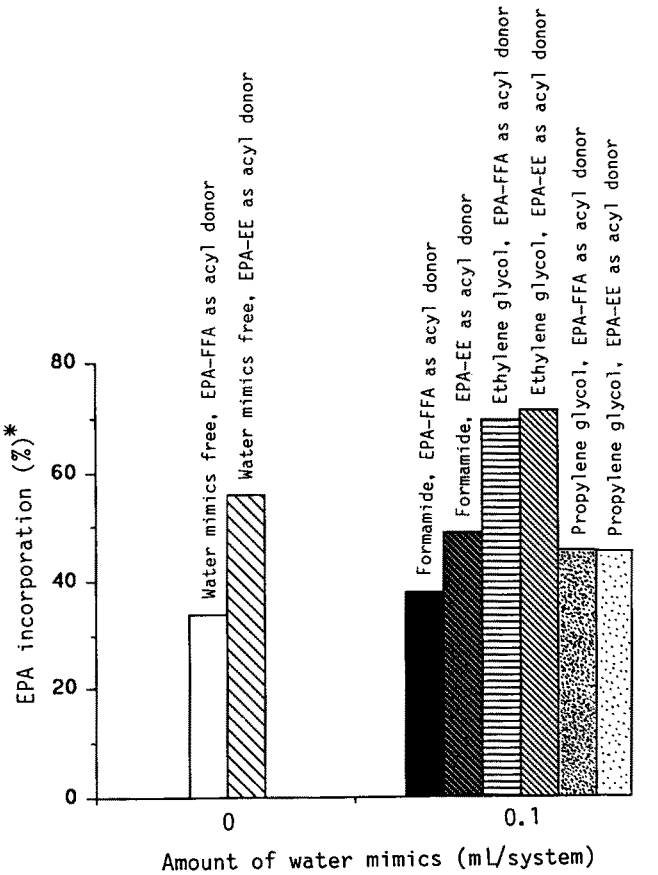

FIG. 1. Effect of water mimics on Lipozyme IM-60-catalyzed acidolysis (Novo Nordisk, Bagsvaerd, Denmark) and transesterification to incorporate eicosapentaenoic acid (EPA) into fish oil triglyceride at water activity of 0.257. Purity of the acyl donors used was 90%. Fish oil triglyceride contained EPA and docosahexaenoic acid (DHA) at 14 and 11%, respectively. *EPA % was obtained from fatty acid composition in the triglycerides. FFA, free fatty acid; EE, ethyl ester.

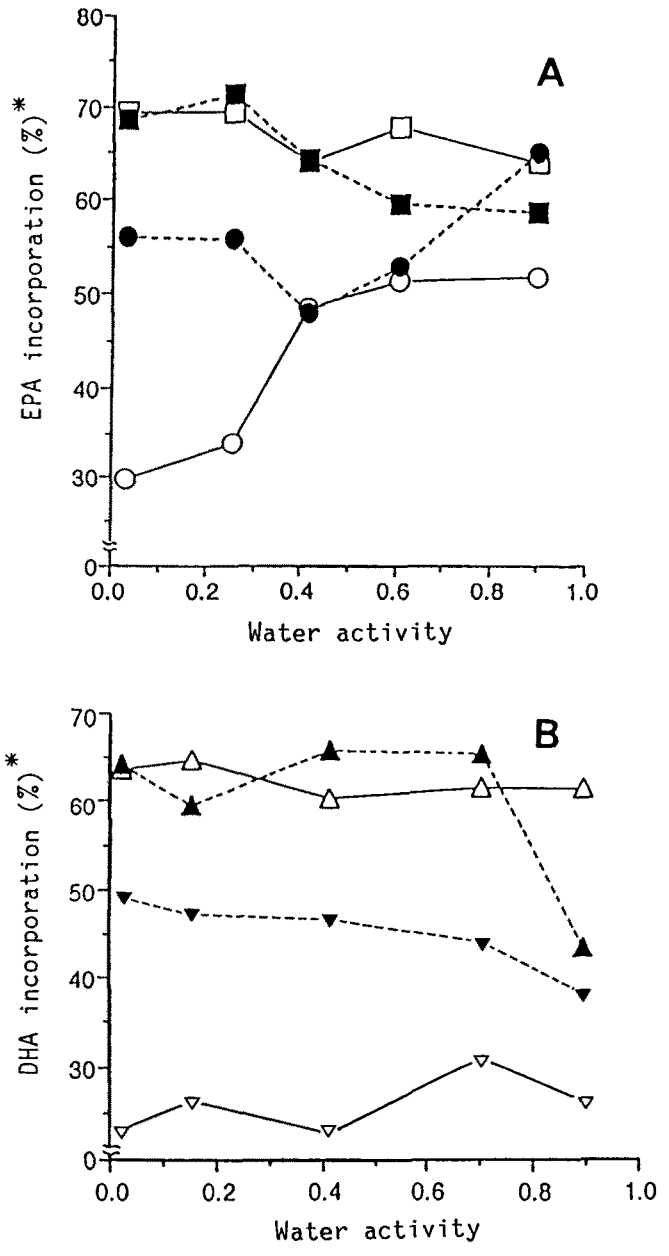

FIG. 2. Effect of ethylene glycol on Lipozyme IM-60-catalyzed acidolysis and transesterification as a function of EPA (A) and DHA (B) incorporation rates in the triglyceride fraction under various water activity conditions. For the reaction conditions, see the Materials and Methods section. Purities of the acyl donors used were 90% for EPA and 88% for DHA. Fish oil triglyceride used, abbreviations, and company location were the same as in Figure 1. *EPA % and DHA % were determined from the fatty acid compositions in the triglycerides. ○, Ethylene glycol-free, EPA–FFA as acyl donor; □, ethylene glycol added (0.1 mL/system), EPA–FFA as acyl donor; ●, ethylene glycol-free, EPA–EE as acyl donor; ■, ethylene glycol added (0.1 mL/system), EPA–EE as acyl donor; ▽, ethylene glycol-free, DHA–FFA as acyl donor; △, ethylene glycol added (0.1 mL/system), DHA–FFA as acyl donor; ▼, ethylene glycol-free, DHA–EE as acyl donor; ▲, ethylene glycol added (0.1 mL/system), DHA–EE as acyl donor.

the TG's EPA content to approximately 70% at low a_w levels (original fish oil TG was 14%) when EPA–FFA was used as the acyl donor. Recovery of EPA-incorporated TG, which can be determined by the TG ratio of the reactant, also increased when ethylene glycol was used. The increase in recovery was larger at all a_w levels when ethylene glycol was used, especially when FFA was the acyl donor (data not shown).

When DHA–FFA (88%) and DHA–EE (88%) were the acyl donors (Fig. 2B), incorporation of DHA into fish oil TG, whose fatty acid composition included 11% DHA, reached 60–67% with ethylene glycol depending on the a_w condition employed. It has been said that it is more difficult to incorporate DHA into TG compared with EPA or other fatty acids. However, the present study has demonstrated that ethylene glycol can strongly promote the incorporation of DHA into TG. Recovery of DHA-incorporated TG, which can be determined by the TG ratio of the reactant, also increased with the addition of ethylene glycol at all a_w levels (data not shown).

Optimum ethylene glycol amounts were briefly examined. Excess amounts of ethylene glycol were deleterious for transesterification between EE and TG, owing to the aggregation of Lipozyme (Fig. 3).

Effect of water mimics on acidolysis to incorporate EPA and DHA into soy PC. As a preliminary study, we examined the effect of a_w on soy PC acidolysis. The higher the a_w, the faster the initial rate of EPA incorporation into position *sn*-1 of soy PC. However, recovery results were completely opposite. Therefore, it seems impossible to satisfy the incorporation rate of EPA and high recovery at the same time.

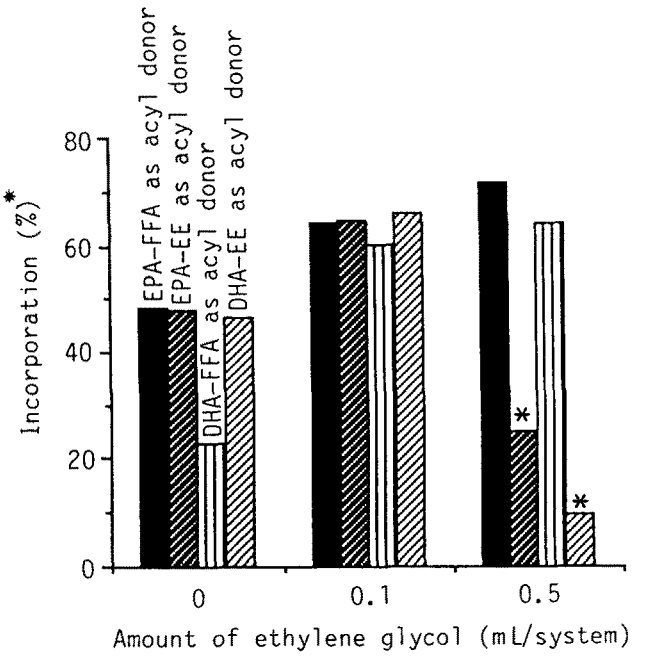

FIG. 3. Effect of ethylene glycol on Lipozyme IM-60-catalyzed acidolysis and transesterification to incorporate EPA and DHA into fish oil triglyceride at water activity of 0.415. Acyl donors and fish oil used were the same as in Figure 2. Large asterisks show the occurrence of aggregation of the Lipozyme. *EPA % and DHA % were determined from fatty acid composition of the triglycerides. Abbreviations and company location as in Figure 1.

We compared the water and mimic effects on EPA incorporation into soy PC. Propylene glycol was the most promising water mimic, owing to its good incorporation of EPA and good recovery at the same time. Though the initial rate of EPA incorporation was low, *N,N*-dimethylacetamide exhibited the highest recovery among the three water mimics examined (data not shown). However, *N,N*-dimethylacetamide is generally recognized as unsafe. Thus, propylene glycol was rated the most favorable water mimic for this reaction. Excess amounts of propylene glycol addition seemed to denature Lipozyme, as illustrated in Figure 4A. It also accelerated the hydrolysis of PC to give poor recovery (Fig. 4B). We examined the combined usage of a little amount of water and propylene glycol (Fig. 5). The results in Figure 5 show satisfactory incorporation of EPA, reaching up to 40% conversion after 48 h reaction, which corresponds to 80% of the theoretical maximum incorporation level (because Lipozyme is known to incorporate the desired fatty acid exclusively into position *sn*-1 of PC). At the same time, this combined usage of propylene glycol and water resulted in a high recovery of 80%.

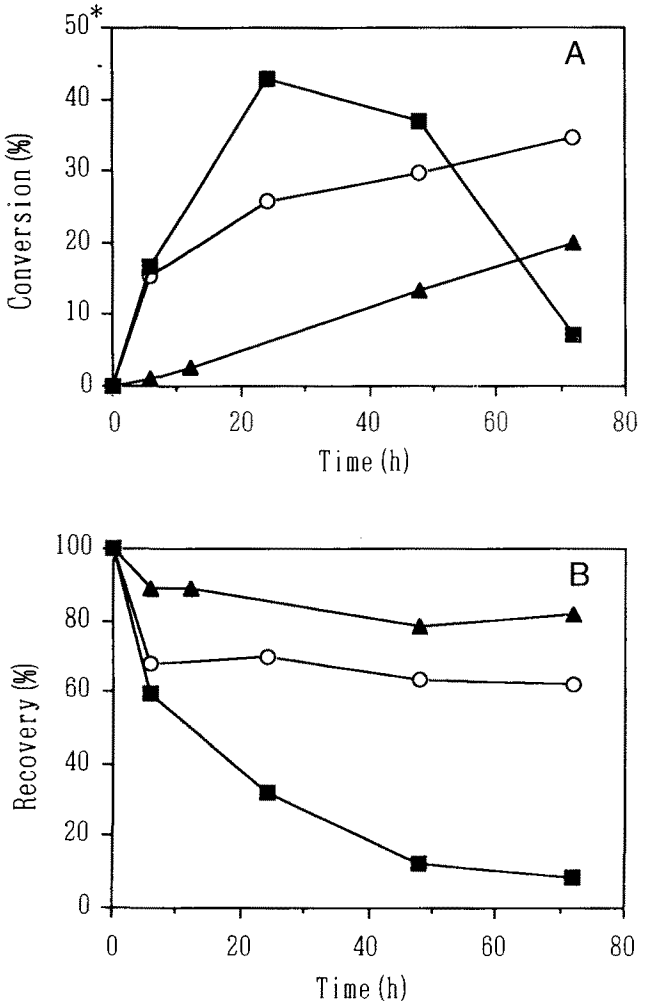

FIG. 4. Effect of propylene glycol and water on Lipozyme IM-60-catalyzed acidolysis to incorporate EPA into soy phosphatidylcholine. Reaction conditions: hexane (0.5 mL) solution containing soy phosphatidylcholine (10 mg), EPA (60 mg) and Lipozyme IM-20 (40 mg by initial weight). ▲, No water mimics, $a_w \simeq 0$; ○, propylene glycol (1.0 μL/system), $a_w \simeq 0$; ■, propylene glycol (2.5 μL/system), $a_w \simeq 0$. *Theoretical maximum of the EPA incorporation corresponds to 50% because Lipozyme exclusively incorporates EPA into position *sn*-1 of the phospholipid. Abbreviations and company location as in Figure 1.

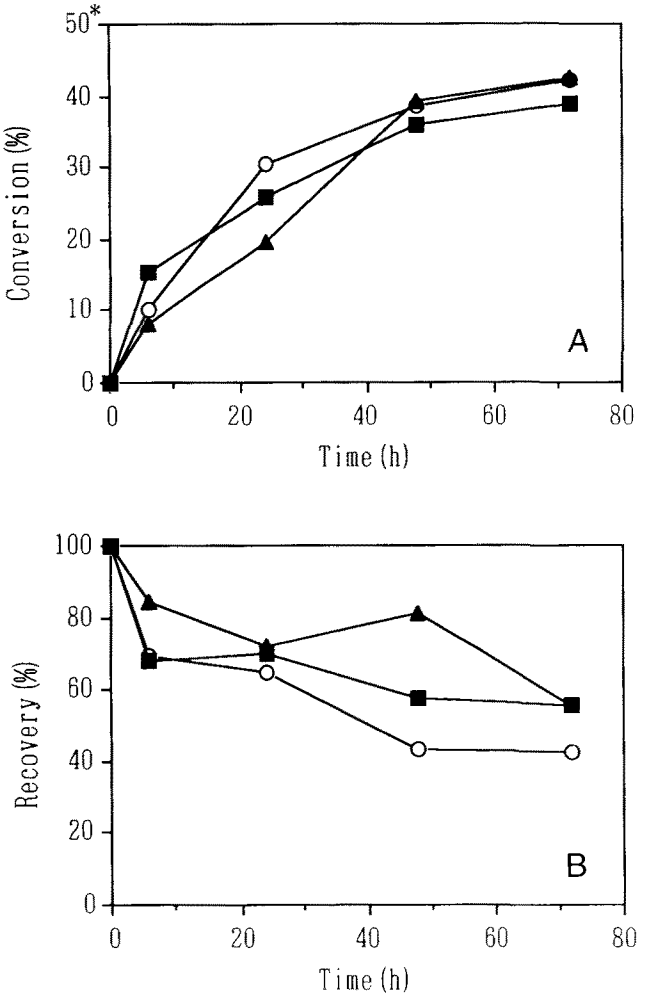

FIG. 5. Effect of combined usage of water and propylene glycol on Lipozyme IM-60-catalyzed acidolysis to incorporate EPA into soy phosphatidylcholine. *Theoretical maximum of EPA incorporation corresponds to 50% because Lipozyme exclusively incorporates EPA into position *sn*-1 of the phospholipid. ○, Water (1.0 μL/system); ■, propylene glycol (1.0 μL/system), $a_w \simeq 0$; ▲, propylene glycol (0.5 μL) with water (0.5 μL). Other reaction conditions were the same as in Figure 4. Abbreviations and company location as in Figure 1.

DISCUSSION

It is hypothesized that the hydration of hydrophilic groups of enzyme protein is the first essential step for lipase activation (5). Consequently, the water of hydration cannot be replaced by nonaqueous solvents. At this a_w level, hydrolysis cannot occur because the lipase protein molecule is assumed to be in a rigid conformation. Above the hydration a_w level, water can act as a lubricant (5), producing a flexible conformation of li-

pase. Thus, flexibility is suggested to be the second essential condition for lipase activity. In addition, water acts as a nucleophilic substrate for hydrolysis and is detrimental to good incorporation of fatty acids and good recovery of glycerolipid at the same time. Our study suggests that the lubricating effect can be replaced by water mimics. (Figs. 2 and 5). We conclude that, in a nonaqueous medium containing appropriate amounts of water and water mimics, Lipozyme activates a net synthesis of EPA and DHA into glycerolipids.

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