Enzymatic Preparation of Glycerides Rich in Docosahexaenoic Acid from Thraustochytrid Single Cell Oils by Candida rugosa Lipase

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Abstract: Two kinds of single cell oils from thraustochytrids including Schizochytrium limacinum SR21 and Thraustochytrium sp. KK17-3 were hydrolyzed with Candida rugosa lipase to prepare the acylglycerols rich in docosahexaenoic acid (DHA, C22 : 6n-3). When the lipase at 800 units/g-oil was employed for a 24-h hydrolysis of SR21 oil at 37°C, DHA content in the glycerides fraction reached at up to 67.3%, being much higher than that in the original oil (40.5%), even in the absence of surfactants. In particular, monoacylglycerol contained DHA at 85.6%. After removal of free fatty acids, the glycerides fraction was further hydrolyzed to improve its DHA content to 81.3%. On the contrary, an incapability to concentrate docosapentaenoic acid (DPA, C22 : 5n-6) in the glycerides suggested that the lipase strictly discriminated in fine structure of respective fatty acids. Although the addition of either Triton X-100 or deoxycholate, but not Tween 20, facilitated the rapid production of monoacylglycerol rich in DHA, its accumulation level was not significantly improved. The lipase was also effective in enriching DHA (63.0%) in glycerides fraction from KK17-3 oil (30.7%). Eicosapentaenoic acid (EPA, C20 : 5n-3) and arachidonic acid (AA, C20 : 4n-6) present in this oil were released from glycerides faster than DPA and DHA by the hydrolysis. The resistance to hydrolysis of the predominant ester bonds composed of the polyunsaturates and palmitic acid (C16 : 0) in the oils from SR21 and KK17-3 was in the following order, DHA > DPA > EPA > AA > C16 : 0. The thraustochytrid oils are therefore suitable source for the preparation of DHA-rich glycerides, especially monoglycerides.

Key words: Candida rugosa lipase, docosahexaenoic acid, Schizochytrium, single cell oil, Thraustochytrium

1 Introduction

Docosahexaenoic acid (DHA, C22 : 6n-3) shows many positive effects on diseases such as hypertension, arthritis, atherosclerosis, depression, thrombosis, and cancers (1). In addition, the development of brain and retina in infants and the maintenance of normal brain function in adults absolutely require DHA (2, 3). These clinical and physiological efficacy of DHA could be due at least partly to its role in regulating gene expression in lipogenic as well as other tissues (4).

Edible DHA is commercially available in forms of both methyl/ethyl ester and triacylglycerol (TG) that was found to be more quickly hydrolyzed and easily
absorbed than the former (5, 6). The conventional TG rich in DHA derived from fish oil source contained a large proportion of saturated fatty acids which were regarded to show adverse effect on cardiovascular disorders (7). The selective hydrolysis of fish oil by lipases from genera Candida, Geotrichum, Rhizopus, and Pseudomonas were effective in concentrating DHA in glycerides composed of TG, diacylglycerol (DG), and monoacylglycerol (MG) (8-11). However, the final DHA content in the undigested glyceride fractions ranged at only 50-55%. This was presumably caused by the lower content of DHA in the original fish oil (about 10% of total fatty acid).

The single cell oils produced by thraustochytrids with two typical genera, Schizochytrium and Thraustochytrium, were found to be the satisfactory alternatives for DHA production (12, 13). In fact, they exhibit simple fatty acid composition with a high DHA content (up to 40% of total fatty acid) and lack peculiar taste of fish oil. Among the reported strains, S. limacinum strain SR21 is regarded to be the most excellent in terms of the DHA productivity due to its high growth rate (14).

In this paper, we attempt to prepare the DHA-rich glycerides by selective hydrolysis of single cell oil from the strain SR21 with C. rugosa lipase. In addition, the hydrolysis behavior and substrate selectivity of the lipase on ester bonds composed of polyunsaturated fatty acids (PUFA) was investigated as compared with the oil from Thraustochytrium sp. KK17-3, which accumulates the other PUFA such as eicosapentaenoic acid (EPA, C20 : 5n-3) and arachidonic acid (AA, C20 : 4n-6) in addition to DHA (15).

2 Experimental

2-1 Materials

S. limacinum SR21 (14) and Thraustochytrium sp. KK17-3 (15) were cultivated in 500-mL baffled flasks containing 100 mL of medium composed of 40 g/L glucose, 10 g/L peptone, and 5 g/L yeast extract at the salinity equivalent to 50% that of seawater at 28°C for 4 d with rotary shaking at 160 rpm. Cells were harvested by centrifugation (5000 rpm, 10 min) and extracted two or three times with chloroform/methanol mixture (2 : 1, v/v) by homogenization of cells in the presence of glass beads ( ~ 0.1 mm in diameter) using a homogenizer (CM-100; Iuchi, Osaka) (16). Total lipid was concentrated by evaporating the mixture with rotary evaporator. C. rugosa lipase (724 units/mg) was purchased from Sigma (St. Louis, MO, USA).

2-2 Standard Hydrolysis Reaction

One gram of oil sample and 2 mL of phosphate buffer (50 mM, pH 7.2) were mixed in a screwed glass tube by sonication for 10 min. Lipase solution (800 units for standard reaction) was added into the mixture and then completely homogenized by vortexing. The tube was flushed with nitrogen and then incubated in a bath shaker at 37°C for 24 h with reciprocal shaking at 150 rpm unless otherwise stated. Control experiment (0-h reaction) was carried out as above but the enzyme had been inactivated by boiling for 15 min. Aliquots of the reaction mixture were collected periodically and each reaction was quenched immediately by adding 10 vol of chloroform/methanol mixture (2 : 1, v/v) followed by mixing.

2-3 Analysis of Lipid Composition of the Hydrolyzed Mixture

After centrifugation (5000 rpm, 10 min) of the hydrolyzed sample, the lower phase was collected and concentrated to 1 mL under the reduced pressure. To separate free fatty acids (FFA), the hydrolysate was titrated by the addition of 20-40 μL of methanolic 0.05 N KOH solution and centrifuged (5000 rpm, 10 min). The chloroform phase containing glycerides fraction was taken out and then concentrated. The hydrolyzed products were separated by thin-layer chromatography (TLC) on a silica gel plate (Kieselgel 60; Merck, Darmstadt, Germany) with benzene/chloroform/acetic acid (70 : 30 : 2, v/v) as developing agent. Triolein hydrolsate mixture (TLC Mix 34; Larodan Fine Chemicals, Malmo, Sweden) was used as the marker. The spots were visualized by spraying TLC plates with 3% CuSO4 in 30% sulfuric acid aqueous solution and heating at 120°C for 20 min, and were quantified by densitograph (AE-6960; ATTO, Tokyo).

2-4 Determination of Fatty Acid Composition on Lipid Classes

To separate lipid classes, the hydrolyzed mixture was applied on a silica gel column (5 × 180 mm; Wakogel C-200, Wako Chemicals, Osaka) and eluted with 20 mL of hexane/diethyl ether (9 : 1, v/v). The eluates were collected as 0.2-mL fractions. After checking the composition of each fraction by TLC, the fractions contain-
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The lipids were hydrolyzed and esterified by treatment with 10% methanolic HCl (Tokyo Kasei Kogyo, Tokyo) at 60°C for 3 h. Fatty acid methyl esters were applied to a gas-liquid chromatograph (GC-17A; Shimadzu, Kyoto) equipped with a flame ionization detector and a split injector (split ratio = 1/25) on a TC-70 capillary column (0.25 mm × 30 m; GL Science, Tokyo) and resolved with temperature programming (190 to 220°C at 2°C/min). The peaks were identified and calibrated with standard fatty acid methyl esters.

3 Results

3.1 Effect of Amount of Lipase on Hydrolysis of SR21 Oil

Single cell oil from S. limacinum SR21 containing TG at 88.7% was hydrolyzed by various amounts of C. rugosa lipase for 24 h. As illustrated in Table 1, the content of unhydrolyzed TG was decreased and the resultant FFA was raised with the increase in lipase activity. When 800 units/g-oil of lipase was used, the total content of glycerides (TG + DG + MG) in hydrolysate was 52.4% and the MG content reached to the maximum (9.2%). Although the higher amount of lipase (1200 units/g-oil) could result in glycerides with DHA at 69.4% that was slightly higher than that in 800 units/g-oil lipase (64.3%), the glycerides content in the hydrolyzed products was only 36.5%.

Lipid classes from the hydrolyzed products at 800 units/g-oil of lipase were investigated for their fatty acid composition (Table 2). It was notable that DHA contents in MG and DG were 85.6% and 73.6%, respectively, whereas docosapentaenoic acid (DPA, C22 : 5n-6) and saturated fatty acids were found at lower contents than those in original oil. On the contrary, free fatty acid fraction did not contain the detectable levels of DHA, which suggested a resistance nature of DHA.

<table>
<thead>
<tr>
<th>Lipase activity (unit/g-oil)</th>
<th>Lipid composition of hydrolysate (wt%)</th>
<th>DHA content in glyceride fractions (wt% of total fatty acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TG</td>
<td>1, 3-DG</td>
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<tr>
<td>0</td>
<td>88.7</td>
<td>2.9</td>
</tr>
<tr>
<td>200</td>
<td>55.6</td>
<td>5.1</td>
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<tr>
<td>600</td>
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<td>5.4</td>
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<tr>
<td>800</td>
<td>20.5</td>
<td>4.5</td>
</tr>
<tr>
<td>1200</td>
<td>11.5</td>
<td>4.2</td>
</tr>
</tbody>
</table>

1) The reaction mixture was composed of 1 g oil, 2 mL phosphate buffer (50 mM, pH 7.2), and indicated amount of C. rugosa lipase. The reaction was performed at 37°C for 24 h with constant agitation. TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid.

<table>
<thead>
<tr>
<th>Lipid fraction</th>
<th>Fatty acid composition (wt% of total fatty acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C14 : 0</td>
</tr>
<tr>
<td>Original oil</td>
<td>2.0</td>
</tr>
<tr>
<td>Glycerides</td>
<td>0.0</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>1.5</td>
</tr>
<tr>
<td>Diacylglycerol (1, 2/-1, 3-)</td>
<td>0.0</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>0.0</td>
</tr>
<tr>
<td>Free fatty acid</td>
<td>4.3</td>
</tr>
</tbody>
</table>

1) The hydrolysates were prepared as in Table 1 using 800 unit/g-oil of C. rugosa lipase.
to be hydrolyzed by *C. rugosa* lipase.

### 3.2 Time Course on Hydrolysis of SR21 Oil

The lipid composition was monitored on a 40-h reaction of the hydrolysis of *S. limacinum* SR21 oil by *C. rugosa* lipase at 800 units/g-oil (Fig. 1). The increase in FFA up to 60% was accompanied with the decrease in TG to less than 20% throughout the hydrolysis course. The contents of 1, 2-DG and 1, 3-DG were maximum at 20-22 h and then decreased gradually till the end of the reaction. It was unexpected that MG did not appear in the hydrolyzed mixture until 20 h when DGs began to decline. The retardation might be attributable to the difference in the accessibilities of glyceride molecules to lipase, as mentioned below.

With the progress in hydrolysis, DHA was concentrated in glycerides fraction in which other fatty acyl species were decayed (Table 3). The enrichment of DHA was obvious before 22 h in particular. However, the increase in DHA content was getting slower after 28 h even though the hydrolysis reaction was still running. This was probably attributed to the inhibition caused by the accumulated FFA that could be re-esterified with glycerides through the reverse reaction catalyzed by the lipase. Thus, the 24-h glycerides fraction free of FFA was prepared as substrate to repeat the hydrolysis treatment for 8 h under the same condition as the first one. Consequently, DHA content was increased to 81.3%, which was even higher than at 40 h in the first reaction (72.3%; Table 3).

DPA as another principal PUFA in SR21 oil could not be enriched in the hydrolysis in which its final content was decreased to 6.3% in glycerides fraction (Table 3). This was equivalent to 65% of that (9.7%) in original oil even though its content was temporarily raised up to 11.5% at the early stage (8 h). On the other hand, C16 : 0 and other saturated fatty acids were steadily removed from the glycerides. It was notable that a larger amount of 1, 2-DG than 1, 3-DG was accumulated in the hydrolysate (Fig. 1). This could be consistent with the observations that the saturated species were mainly located at sn-1 or sn-3 position of TG molecules carrying PUFA in sn-2 position (17).

### 3.3 Effect of Adding Surfactant on Hydrolysis of SR21 Oil

In the above hydrolysis course (Fig. 1), MG was generated with a lag time (0-18 h) when DGs were accumulated substantially at 34% in the hydrolysate. Since DGs contained 19.0% of saturated fatty acids at 24 h of the reaction (Table 2), MG should be promoted to appear in the earlier stage through the hydrolysis of such DGs. Due to that the hydrolysis of lipids by lipase takes place on a hydrophobic-hydrophilic interface, the emulsion status between oil and water phases occasionally affects the hydrolysis progress.

Accordingly, ionic and non-ionic surfactants were
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tested for their effects on hydrolysis of SR21 oil by the lipase. As shown in Fig. 2, Triton X-100 could facilitate the hydrolysis of TG resulting in the rapid generation of DG, MG, and FFA. However, the accumulation of MG reached to only 9.8% at 20 h, which was equivalent to that (9.2%) at 24 h of the reaction free of any surfactants (Fig. 1). Deoxycholate exhibited a similar result while Tween 20 did not show considerable effect (data not shown).

3.4 Hydrolysis of KK17-3 Oil

Lipids from Thraustochytrium sp. KK17-3 was comprised of more than 92.4% of TG that contained AA, EPA, and monounsaturated fatty acids in addition to C16 : 0, DPA, and DHA (see line 1 of Table 4). As given in Fig. 3, C. rugosa lipase showed a hydrolysis pattern for KK17-3 oil similar to that for SR21 oil. However, the levels of the temporary accumulation of DGs (18-22 h) and the generation of MG at the late phase (28-40 h) were much less than those in the case of SR21 oil. These could be due to that the total content of fatty acids other than DHA, which was sensitive to the attack by the lipase, was 69.3% in original KK17-3 oil, being higher than in SR21 oil (59.5%). In fact, some halves in the contents of fatty acids except DHA were eventually removed from glycerides fraction at 40 h in the hydrolysis reaction (Table 4). Similarly to the SR21 oil, only DHA exhibited the resistance against the hydrolysis by C. rugosa lipase and less than 2% of DHA was found in FFA fraction of the hydrolysate at the end of the reaction (data not shown).

3.5 Evaluation of Hydrolysis Resistance of Fatty Acids in Single Cell Oils

In both cases of the hydrolysis of oils from S. limacinum SR21 and Thraustochytrium sp. KK17-3, the DHA enrichment in glycerides fraction was accompa-

![Fig. 2](image-url) Effect of Surfactants on Hydrolysis of S. limacinum SR21 Oil by C. rugosa Lipase. Triton X-100 was added to the hydrolysis reaction mixture at 1% (w/v). Abbreviations are the same as in Fig. 1.
nied with the removal of the scissile fatty acids (Tables 3 and 4). This could be regarded as the selective hydrolysis which was dependent on the aimed ester bond resistance to \( C. \ rugosa \) lipase. Since this lipase is non-specific for stereo-specific position of ester bonds on glycerol backbone (18), we estimated its specificity for each fatty acyl segment using hydrolysis resistance value that could reflect the difficulty of the target to be hydrolyzed (9).

In the case of SR21 oil, \( C16 : 0 \) showed the weakest hydrolysis resistance to the lipase whereas DHA proved to be the highest as depicted in Fig. 4A. It was noted that none of the ester bond composed of DHA was hydrolyzed at 28 h when up to 90% of \( C16 : 0 \) was removed from glycerides. DPA indicated the intermediate values throughout the reaction, and therefore the hydrolysis resistance for each predominant fatty acid was following the order: DHA \( \gg \) DPA \( > \) \( C16 : 0 \). On the other hand, the order of the hydrolysis resistance for KK17-3 fatty acids at any time points was as follows: DHA \( \gg \) DPA \( > \) EPA \( > \) AA \( > \) \( C16 : 0 \) (Fig. 4B).

It should be pointed out that, even in the presence of surfactant, DPA in KK17-3 oil showed a little stronger resistance to be hydrolyzed compared to that in SR21 oil. For example, hydrolysis resistance value for DPA in KK17-3 at 20 h was 86.3% that was higher than 75.4% in SR21. The difference was probably caused by the presence of AA, EPA, and monounsaturated fatty acids which might delay the hydrolysis of DHA and DPA in KK17-3 oil. Therefore, in view of the effective enrichment of DHA in DGs and MG, it should be desirable to use source oils with simple fatty acid composition and higher content of DHA, such as the single cell oil from \( S. \ limacinum \) SR21.

4 Discussion

Our results indicated clearly that the ester bonds comprising of shorter acyl chain (C14-18) were hydrolyzed faster than those of longer ones (C20-22) by \( C. \ rugosa \) lipase. This could result from the different mode in the recognition of the respective substrates by the enzyme. Lipases are discriminated from each other in

<p>| Table 4 Fatty Acid Composition of Glycerides Fraction on Time Course for Hydrolysis of KK17-3 Oil. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
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<th>Hydrolysis time (h)</th>
<th>C15 : 0</th>
<th>C16 : 0</th>
<th>C16 : 1</th>
<th>C18 : 0</th>
<th>C18 : 1</th>
<th>C20 : 4n-6</th>
<th>C20 : 5n-3</th>
<th>C22 : 5n-6</th>
<th>C22 : 6n-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>26.6</td>
<td>7.6</td>
<td>2.2</td>
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<td>9.6</td>
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</tr>
<tr>
<td>8</td>
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<td>1.1</td>
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<td>8.1</td>
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<td>0.9</td>
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<td>3.4</td>
<td>7.9</td>
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</tr>
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<td>4.2</td>
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<td>6.4</td>
<td>3.4</td>
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</table>
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their distinctive binding site for acyl fatty acid in an ester substrate, which in fact reflects to the enzymatic specificity for substrate (19), though lipases have a similar principal architecture composed of α/β-hydrolase folds and a Ser-His-Glu catalytic triad in their active sites (20). The C. rugosa lipase has an extremely narrow tunnel-like binding site for the acyl fatty acid that protrudes through the entire lipase molecule (21). In addition, its active site is located much deeper in the protein interior than the active sites of lipases of, for instance, Humicola lanuginose and Rhizomucor miehei (21). The properties of C. rugosa lipase on its molecular structure might not allow to easily harbor the long acyl chain including C22 acids (19, 22). Since the binding of lipase with substrate oil is the first limiting step in hydrolysis reaction (8), the time required for each fatty acid attaching the site determines to some extent their order to be hydrolyzed. Accordingly, it is considered in the case of C. rugosa lipase that the fatty acids with short carbon chain are liable to intrude into the binding site than fatty acids with long carbon chain.

On the other hand, C. rugosa lipase apparently discriminated DHA against EPA in the reaction system even though their carbon chain lengths were the same. This could be ascribed to a higher-order structure of the whole fatty acid molecule arised by the presence of double bonds. The introduction of a carbon-carbon cis double bond in the acyl chain causes a bending of the carbon backbone at about 30° (19). Because there are six cis double bonds in the DHA molecule, the terminal methyl group of the fatty acid situates very close to the ester bond of the glyceride (23). Such configuration could protect against hydrolysis of the ester linkage by lipase through a steric hindrance effect. Therefore, fatty acids with less number of double bonds, such as EPA and saturated fatty acids, might become preferential targets of the lipase.

The 1, 2-DG (including 2, 3-DG) content was much higher than 1, 3-DG throughout the hydrolysis course in both SR21 and KK17-3 oils by C. rugosa lipase lacking specificity to a particular ester bond position (Figs. 1 and 3). This indicated that more DHA moieties were located on the sn-2 position of TG molecule in the source oils, which was not easily removed by the hydrolysis. The occurrence of DHA at the sn-2 position of the 1, 2-DG would result in the generation of 2-MG with DHA by a further hydrolysis of DG at the sn-1 position by lipase. Thus, the thraustochytrid oils containing a higher amount of DHA than the fish oil (12-14) are the better source of the 2-MG carrying DHA (at over 90%), which could be applicable in producing specific glycerides such as symmetrically structured TG (11, 24).

The addition of surfactant was aimed to improve the emulsion of substrate and water since the catalytic reaction occurs on the internal interface between water and

![Fig. 4](image-url)
the oil. It has been reported that the conformational change in *Staphylococcus aureus* lipase induced by Triton X-100 could accelerate the hydrolysis rate (25) and that the non-ionic detergent (pentaoxyethylene octyl ether) increased the activity of *H. lanuginosa* lipase by opening a surface lid directly over an active center (26). In our reaction systems, use of either Triton X-100 or deoxycholate facilitated the rapid production of DG and MG (Fig. 2). This was possibly related to its altering the conformation of *C. rugosa* lipase that also has a lid over its active site (19). Despite Tween 20 structurally similar to Triton X-100, their distinctive extinction coefficients were likely to cause difference between their surface pressures of the resultant phase interface composed of water, oil, and surfactant. In fact, the interface pressure was reported to affect lipase activity and specificity for substrates (27). However, we concluded that the use of surfactant was not effective in terms of high levels of MG production since the maximum MG content at 20 h in the presence of Triton X-100 was only 9.8%, which was not significantly greater than the 24-h product in the reaction in the absence of surfactant (Fig. 1). This could be explained by a possibility that the addition of surfactant promoted the hydrolysis of MG as well as TG and DG, resulting in the release of higher amount of FFA as in fact observed.

Compared with DHA concentrates derived from the hydrolysis of fish oil (9, 10, 28-30), much higher DHA concentration occurred in the hydrolysis of single cell oils from both *S. limacinum* SR21 and *Thraustochytrium* sp. KK17-3 (65-90%; Tables 3 and 4). However, it could not be ignored that the hydrolysis of thraustochytrid oils showed slower decrease rate of TG, a lag time for MG production, and lower accumulation of FFA before 20 h of the reaction (Figs. 1 and 3) in comparison with a hydrolysis of fish oil under the same condition (data not shown). This retardation was not fully eliminated by the use of surfactants (Fig. 2). We consider that this is mainly due to the TG composition with DHA that accounts for up to 60% of total TG from thraustochytrid oils (31), which is significantly higher than that in fish oil (~20%) (32). In fact, not only the ester bonds with DHA exhibits a strong resistance to *Candida* lipase but also the presence of DHA in a substrate molecule reinforces the resistance of the other ester bonds even they are composed of saturated fatty acids (33). After 20 h of the reaction, the change in the composition of TG and DG might improve the status of surface of water and oil profitable to the action of lipase, which resulted in the generation of MG. In addition, there may be another possibility that an unknown substance in thraustochytrid oils could favor the transesterification of DG back into TG rather than the hydrolysis of TG in the early reaction period.

Finally, preparation of glyceride rich in DHA by the enzyme selective hydrolysis on single cell oil in the mild conditions (37°C, pH 7.2, and less chemicals) could reduce the destroy of PUFA molecules, which unavoidably occurred by traditional hydrolysis methods involving extreme of pH and high temperature.

**References**

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231, 15-22.