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Synthesis of Monoacylglycerol Rich in Polyunsaturated Fatty Acids from Tuna Oil with Immobilized Lipase AK

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Abstract

The aim of this study is to produce monoacylglycerols (MAG) rich in polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by glycerolysis of tuna oil with Lipase AK from Pseudomonas fluorescence immobilized on Accurel EP-100 (IM-AK). tert-Butyl methyl ether (MTBE) was the most suitable organic solvent after screening a list of different solvents and their mixtures. The optimum condition for MAG production was found to be 10 %w/v of tuna oil in MTBE, the mole ratio of glycerol to tuna oil 3:1, water added 4 wt% in glycerol, and the amount of IM-AK 30 wt% based on tuna oil. The temperature was controlled at 45°C. Under these conditions with 24 h reaction, the yield of MAG was 24.6% but containing 56.0 wt% PUFA (EPA and DHA). Stability of the IM-AK was also studied. The hydrolytic activity of the enzyme remained 88 and 80% of initial activity after incubated in MTBE for 24 h at 4 and 45 °C, respectively. The $K_m$ and $V_{max}$ values of the lipase-catalyzed glycerolysis of tuna oil in MTBE were found to be 19.47mM and 2.71mgMAG/min, respectively, for IM-AK.

Key words: Monoacylglycerols, glycerolysis, polyunsaturated fatty acids (PUFA), Immobilized lipase, Lipase AK, tuna oil
Introduction

Tuna oil is currently one of the major sources of polyunsaturated fatty acids (PUFA), especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The oil contains approximately 5.7% EPA and 18.8% DHA which are distributed in mixed triacylglycerols (TAG) with ordinary fatty acids (Wongsakul, Prasertsan, Bornscheuer and H-Kittikun, 2003). PUFA have received much attention in recent years because of the health benefits including reduced risk of coronary disease, prevention of certain cancers, and improved immune functions (Narayan, Miyashita and Hosakawa, 2006; Ruxton, Reed, Simpson and Millington, 2004).

Monoacylglycerols (MAG) or mixtures with diacylglycerols (DAG) account for approximately 75% of the emulsifier production and have various applications in different fields (Bornscheuer, 1995; Damstrup, Jensen, Sparsø, Kiil, Jensen and Xu, 2005). MAG are nonionic emulsifiers widely used in bakery products, margarines, diary products, confectionary because of their emulsifying, stabilizing and conditioning properties. Moreover, MAG are also used in pharmaceutical as binders in tablets and as emollients for transdermal, slow-release drugs. Due to the worldwide importance of MAG and their derivatives as surface active additives in a wide range of foods, considerable attention has recently been paid to improve the synthesis of MAG. They are also of great interest in synthetic organic chemistry where they are utilized as synthetic intermediates and as chiral building blocks (Monteiro, Nascimento and Ninow, 2003).

Chemically, MAG can be synthesized at high temperatures using several metallic catalysts. Commercial MAG are widely manufactured by glycerolysis of fats and oils. The glycerolysis reaction is accelerated by the use of inorganic alkaline catalysis, such as NaOH or Ca(OH)\(_2\) at 220-260\(^\circ\)C. However, this leads to a number
of unwanted side products and the reaction occurs in a random manner so that extensive purification of products is required (McNeill & Yamane, 1991; Yang, Rebsdorf, Engelrud and Xu, 2005; Damstrup et al., 2005). Furthermore, the high-temperature chemical process is not suitable for the production of heat-sensitive MAG containing PUFA, in particular from fish oils. Production of heat-sensitive MAG is, however, of great commercial interest owing to their nutritive value, which could be applied in foods and pharmaceuticals (Damstrup et al., 2005; Kaewthong & H-Kittikun, 2005; Yang et al., 2005).

Due to disadvantages of the conventional process, the use of enzymes as catalysts, thus, seems to be a potential as well as imperative alternative for practical considerations. The much lower temperature used (less than 80°C) improves product quality and makes production of heat-sensitive MAG feasible. Lipases have shown a good stability and activity in hydrophobic solvents for MAG synthesis by glycerolysis (Damstrup et al., 2005; Kristensen, Xu and Mu, 2005).

Several studies have dealt with the synthesis of MAG enriched in PUFA such as alcoholysis of triacylglycerols with ethanol using a 1,3-regiospecific lipase or glycerolysis of fish oil using an immobilized lipase (Sakiyama, Toshimi, Tanaka, Osaki and Nakanishi, 2001; Wongsakul et al., 2003; Yang et al., 2005).

Glycerolysis of triacylglycerols (TAG) with lipase in the liquid phase typically yields only 30-50% MAG (Bornscheuer, 1995; Kaewthong & H-Kittikun, 2005). Some authors improved their systems by carrying out the reaction first in a liquid state, but reducing the temperature to crystallize the formed MAG. This led to a shift in the reaction equilibrium so that yields increased to 70-90%. However, continuous production of MAG by this method was impossible (Kaewthong & H-kittikun, 2005). For the bioconversion of various lipophilic or water-insoluble compounds, it is
essential to introduce organic solvents into reaction systems to improve the solubility of these reactants. Furthermore, use of suitable solvents system will result in more homogeneous system and enhance the conversion of substrate, the reaction rate, and the production distribution in favor of MAG formation as well as PUFA content in MAG.

In this work, under the decision of enzymatic glycerolysis with tuna oil as substrate, a list of solvents were first evaluated for the synthesis of MAG. Optimal reaction parameters were investigated in order to obtain MAG in high yields as well as with high PUFA (defined as EPA and DHA) content. Major focus was given to the enrichment of EPA and DHA in the MAG fraction.

Materials and methods

Materials

Crude tuna oil, with water content of 4.7%, was provided from Chotiwat Industrial Co. Ltd (Hat Yai, Thailand). The crude oil was obtained from skipjack tuna heads by a conventional pressing method. The refined oil was achieved through degumming, neutralization, bleaching, and deodorizing. Lipase AK, from *Pseudomonas fluorescens*, with water content of 0.04%, was a gift from Amano (Nagoya, Japan). Microporous polypropylene powder, Accurel EP-100 (particle size < 400μm) was a gift from Akzo Nobel (Oberburg, Germany). All other chemicals and solvents used were of reagent grade or analytical grade.

Hydrolytic activity of lipases

Hydrolytic activity of the immobilized lipase was determined by the modified cupric acetate method of Lee and Rhee (1993). One unit of hydrolytic activity was defined as
the amount of enzyme which liberates 1 μmol equivalent of palmitic acid from palm olein in 1 min at 30°C.

Preparation of immobilized lipase

Accurel EP-100 (10 g) was added to 100 ml of 0.1 M phosphate buffer (pH 7) containing app. 100 U/ml Lipase AK and the reaction mixture was stirred with a magnetic bar at 100 rpm for 30 min. Afterward, 100 ml of 0.1 M phosphate buffer (pH 7) was added and the suspension was filtered through a Buchner funnel by vacuum. The immobilized enzyme was washed with 100 ml of the buffer to remove soluble enzyme and dried in a vacuum desiccator. The immobilized Lipase AK on Accurel EP-100 (IM-AK) was stored at 4°C for further uses.

Glycerolysis reaction

The initial glycerolysis experiments were carried out in a batch system. The reaction mixture consisted of IM-AK (water content 3.0%) 0.6 g, glycerol (99.5%, water content 0.2%) 1.78 g, and 20 ml of 30% (w/v) of tuna oil in organic solvents. Extra 4 wt% water based on the glycerol was added directly to glycerol. The temperature was controlled at 45°C. The reaction was mixed on the shaker at 300 rpm. Samples of the reaction mixture were centrifuged to remove IM-AK before analysis.

Analysis of glycerides by TLC-FID

The components of oil phase were analyzed with a thin-layer chromatography and flame ionization detector (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc. (Tokyo, Japan) for the content of TAG, 1,2(2,3)-DAG, 1,3-DAG, MAG and free fatty acids (FFA) (Kaewthong & H-kittikun, 2005). The sample diluted in chloroform/methanol (2:1 v/v) was spotted onto the chromarod and developed for 35 min in a mixture of benzene/chloroform/acet acid (50:20:0.7 v/v/v) as developing solvent. After development and drying, the rods were subjected to scanning with FID.
Standards were used to identify the peaks. The peak areas were normalized and used for evaluation of the reactions.

**Analysis of fatty acids compositions**

The fatty acid compositions of glyceride species were determined by converting into fatty acid methyl esters (FAME) followed by GC analysis. After evaporating excessive solvent of the sample, the mixture was applied to the normal TLC-plate with silica gel and developed in benzene/chloroform/acetic acid (50:20:0.7 v/v/v). After drying, the MAG band was scraped off and methylated with 0.5%NaOH in methanol (1000 μl) for 10 min at 60°C. The methyl esters were extracted with n-hexane (300 μl) for 1 min. The n-hexane layer was washed with 200 μl distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with a Perkin-Elmer Autosystem XL-GC gas chromatograph (Perkin-Elmer, Norwalk, CT) on a FFFAP column (PERMABOND-FFFAP DF-0.25, 25m × 0.25mm i.d., MACHEREY-NAGEL, Germany). The carrier gas used was helium at a flow rate of 0.5 ml/min (15 psi) and operated in a spit mode with a spit ratio of 50:1. The temperature was started from 150°C for 0.50 min and increased at the rate of 4°C/min to 170 °C, followed with the rate of 5°C/min to 195°C, and further with the rate of 10°C/min to 215°C. the temperature was kept at 215°C for 14 min. Injector and detector temperatures were 250°C (Joseph & Ackman, 1992). Response factors were determined using a standard mixture of fatty acid methyl esters.

**Regiospecific analysis**

The regiospecific analysis of tuna oil was conducted by Grignard degradation with allylmagnesium bromide followed by isolation, methylation, and GC analysis (Soumanou, Bornscheuer and Schmid, 1998; Wongsakul et al., 2003).

**Karl Fischer water content determination**


The water content in the tuna oil, the immobilized lipase, and glycerol as well as in the solvents was determined by Karl Fischer method (720 KFS Titrino, Switzerland, using HYDRANAL titrant and solvents) (Xu, Fomuso and Akoh, 2000).

Statistical analysis

The SPSS program was used for data analysis (SPSS, 1989-2001). Analysis of variance and t-test were used to evaluate the significance and difference of data. Values were considered significant at $P<0.05$ level.

Results and discussions

1. Screening of solvents for the enzymatic glycerolysis of tuna oil.

To select the most suitable solvent for the glycerolysis reaction system, the effect of organic solvents on the catalytic activity of the lipase was examined. The glycerolysis of tuna oil with IM-AK as biocatalyst was carried out in acetone, hexane, isoctane, tert-butyl methyl ether (MTBE) and their combinations. The results are shown in Fig. 1. It was found that MTBE gave the highest yield of MAG at 20.4 wt% with yield of PUFA (EPA and DHA) about 14.8 wt%. Previously, Kaewthong and H-Kittikun (2005) used the combination of acetone/isoctane mixture (3:1, v/v) as solvent for glycerolysis of palm olein. Wongsakul et al. (2003) used acetone as organic solvent for alcoholysis of tuna oil by Lipase PS-C. Moreover, Chang and Rhee (1991) used isoctane as organic solvent for continuous glycerolysis of olive oil in CSTR. Therefore, the selection of solvent seems affected by many different issues. As information collected so far, it is strongly dependent on the selection of lipases. Other issues such as oil type, reactor selection, and reaction mechanism might have effect as well. As to this study, we decided to use MTBE for further study.
2. Effect of water content

Water content is recognized as an important factor in transesterification reactions. A certain amount of water is necessary to preserve the catalytically active conformation of the enzyme and to allow the formation of an acyl-enzyme complex. In contrast, excessive water causes acyl migration lead to decrease in MAG yield (Wongsakul et al., 2003). Therefore, optimal water content is the foremost important factor that should be sorted out in the quite hydrophilic solvent. Initial water content of the glycerol in the range of 4-12 wt% was studied. The results are shown in Fig 2. The highest yield of MAG of 20.7 wt% contained 15.7 wt% PUFA was obtained when 4% the water was added in glycerol. When more than 4 % water was added, the yield of MAG dropped gradually. This may be due to hydrolysis. Yamane, Kang, Kawahara and Koizumi (1994) found that FFA content at equilibrium depended on the water concentration in the glycerol phase. This can eventually lead to the decrease of MAG yields as early mentioned.

3. Effect of substrate concentration in MTBE

In a solvent system, the concentration of substrate will eventually affect the reaction rate based on Michaelis-Menten kinetics even though solvent can help create a homogeneous system. In order to select an efficient initial substrate (tuna oil) concentration for glycerolysis, the effect of tuna oil concentration was investigated. The results are shown in Fig. 3. The MAG yield increased with increasing the concentration of tuna oil as well as PUFA content in MAG increased when tuna oil concentration was increased. At the concentration of 10 %w/v tuna oil in MTBE with the mole ratio of glycerol to tuna oil about 3:1, the best yield of MAG at 22.1 wt% with PUFA content about 38.5 wt% was obtained after 24 h incubation. When the concentration of tuna oil was lower than 10 %w/v, the yield of MAG was decreased.
Solvent plays multiple roles and has more than one function in the system. It is first to make a homogeneous system and increase mass transfer by reducing the viscosity of the system. One the other hand, solvent may increase inhibition to the lipase since it deprives off water from the lipase structure. Certainly addition of solvent decreases the amount of available substrate at the interface between the solvent and glycerol and hence decreases the MAG yield. The amount of glycerol also plays a role in reactions and the system. Glycerol in the mixture would create difficulty for the reaction system if without solvent. On the other hand, Yang and Rhee (1991) suggested that glycerol could act as an effective stabilizer against thermal and solvent denaturation. However, Bornscheuer and Yamane (1994) showed that the optimum mole ratio of glycerol to palm olein for MAG production in the solid-phase system was 2.7:1 where at lower glycerol to TAG mole ratio (1:2), the main product of glycerolysis was diacylglycerols. Therefore, the amount of glycerol affected also the reaction equilibrium. In the present study, 10 %w/v of tuna oil with ca. 3 fold glycerol addition in moles gave the optimal system of the glycerolysis reaction.

4. Effect of IM-AK loading

The effect of IM-AK loading on MAG production was determined. The results are shown in Fig 4. When increasing the amount of IM-AK in the reaction mixture, the MAG production was also increased. However, no benefit came from increasing IM-AK above 30 wt% of tuna oil. Therefore, the amount of IM-AK 30 wt% of tuna oil was used for further study.

5. Effect of temperature

Temperature plays two roles in the reaction system. Firstly, higher temperature can reduce the viscosity as well as improve the substrate diffusion or its solubility. Secondly, enzymes usually have a temperature optimum. Therefore, an optimal
temperature should be selected in terms of the overall performance of the reaction.

The effect of temperature (30-50°C) on MAG production from tuna oil was studied. When temperature was controlled in 30-45°C, the MAG production increased with increasing temperature (data not shown). This result was a consequence of the increase in the reaction rate. In contrast, when increasing the temperature from 45 to 55°C the yield of MAG was decreased. The temperature of 45°C was considered an optimal temperature for the reaction system.

6. Stability of IM-AK in MTBE mixture

Stability of IM-AK in MTBE was studied at 4 and 45°C. The results show that more than 88 and 80% of hydrolytic activity remained after incubation for 24 h, respectively (data not shown). However, Fukui, Kawamoto, Sonomoto and Tanaka (1990) found that benzene was better for lipase stability while gave a moderate result for lipase activity. Kang and Rhee (1989) suggested that the immobilized lipase activity in a reverse-phase system decreased as the polarity of solvent increases. Kwon, Han and Rhee (1995) reported that the enzyme was stabilized by the substrate in a two-phase reaction system (isooctane-water); the half-life of the enzyme was 10 h without the substrate and 20 h with 30% olive oil at 30 °C. Stability is a very complicated issue for many lipases. It not only relates to the characteristics of a lipase but also relates to the reaction system selected. More work is needed to improve the stability of the lipase used.

7. MAG production under optimal conditions

The optimal conditions for MAG production were decided as tuna oil concentration of 10 %w/v in MTBE, the mole ratio of glycerol to tuna oil about 3:1, water content in glycerol with 4 wt% and using IM-AK 30 wt% of tuna oil. The temperature was controlled at 45°C. The reaction time course is given in Fig. 5. The
yield of MAG was 24.5 wt% and PUFA (EPA and DHA) content was 56.0 wt% after 24 h incubation.

The reaction products were separated by TLC. The fatty acid profiles of each band (glyceride species) were determined by GC (Table 1). As shown, monodocosahexylglycerol and monooleylglycerol were the predominant MAG in the products.

8. Kinetics of the glycerolysis using both Lipase AK and IM-AK

The kinetic constants ($K_m$ and $V_{max}$) for glycerolysis of tuna oil with the non-immobilized Lipase AK as well as its immobilized form (IM-AK) were determined in MTBE by measuring initial reaction rates with varying amount of tuna oil (50-500mM). The results are shown in Fig 7. The values of the kinetic constants were obtained from Lineweaver-Burk plot. $K_m$ and $V_{max}$ of the original Lipase AK were 39.26 mM and 11.38 mgMAG/min, respectively; while $K_m$ and $V_{max}$ of IM-AK were 19.47 mM and 2.71 mgMAG/min, respectively. IM-AK had smaller $K_m$ and $V_{max}$ values than its original form, meaning the catalytic capacity of the immobilized form was reduced. The potential allowed substrate concentration is also reduced. A similar result was obtained in hydrolysis of olive oil by Candida rugosa lipase (Montero, Blanco, Virto, Landeta, Agud and Solozabal, 1993). In general, the immobilization of biocatalysts can lead to an activity reduction. It can also cause diffusional limitation of substrates in the immobilized biocatalyst system.

Conclusions

Glycerolysis of tuna oil was investigated to produce MAG rich in PUFA using immobilized Lipase AK. The optimum conditions for MAG production were found to be 10 %w/v of tuna oil in MTBE, glycerol to tuna oil ca. 3:1 mol/mol, water added in
glycerol was 4 wt% and the amount of IM-AK used was 30 wt% of tuna oil. The
temperature was controlled at 45°C. Under these conditions, the yield of 24.6 wt%
containing of 56.0 wt% PUFA (EPA and DHA) was obtained at 24 h. MAG were
produced in good yield with high content of PUFA, especially, EPA and DHA. Thus,
a suitable product or starting material for synthesis of structured triglycerides can be
obtained.

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lipases, as well as, Akzo Nobel for Accurel EP-100.
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Table 1. Fatty acid compositions of species of tuna oil and reaction product after TLC separation

<table>
<thead>
<tr>
<th>composition</th>
<th>wt%</th>
<th>Fatty acid composition (wt%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C14:0</td>
<td>C16:0</td>
</tr>
<tr>
<td>Fish oil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAG</td>
<td>99.3</td>
<td>4.2</td>
</tr>
<tr>
<td>MAG</td>
<td>0.4</td>
<td>14.6</td>
</tr>
<tr>
<td>FFA</td>
<td>0.3</td>
<td>14.1</td>
</tr>
<tr>
<td>sn-2**</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<tr>
<td>1,2-DAG</td>
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</tr>
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</tr>
<tr>
<td>MAG</td>
<td>24.5</td>
<td>1.7</td>
</tr>
<tr>
<td>FFA</td>
<td>41.6</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*Major fatty acids identified.

**Fatty acids composition of tuna oil at the sn-2 position before glycerolysis.
Figure captions:

Fig. 1. Screening of organic solvents on MAG production with IM-AK. The reaction mixture contained 20 ml of 30 %w/v tuna oil in organic solvents and 1.78 g glycerol with 4 wt% water. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45°C for 24 h. PUFA-polyunsaturated fatty acids, here meaning EPA and DHA and MAG-monoacylglycerol. The Y-axis indicates the MAG weight content in the normalized lipid species profile analyzed by TLA-FID and PUFA weight content in the normalized fatty acid composition of the MAG fraction analyzed by GC.

Fig. 2. Effect of water content in glycerol on MAG production by IM-AK. The reaction mixture contained 20 ml of 30 %w/v tuna oil in MTBE and 1.78 g glycerol with various amounts of water content. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45°C for 24 h. Abbreviations and notes to Y-axis see Figure 1.

Fig. 3. Effect of fish oil concentration on MAG production by IM-AK. The reaction mixture contained various amounts of tuna oil in 20 ml of MTBE and 1.78 g glycerol with 4 wt% water. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45°C for 24 h. Abbreviations and notes to Y-axis see Figure 1.

Fig. 4. Effect of IM-AK loading on MAG production. The reaction mixture contained 20 ml of 10 %w/v tuna oil in MTBE and 1.78 g glycerol with 4 wt% water. The reaction was carried out at 300 rpm and 45°C for 24 h. Abbreviations and notes to Y-axis see Figure 1.
Fig. 5. Time course of glycerolysis by IM-AK in MTBE. The reaction mixture contained 20 ml of 10 %w/v tuna oil in MTBE and 1.78 g glycerol with 4 wt% water. The amount of IM-AK was used 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45°C for 24 h. Abbreviations see Figure 1.

Fig. 6. Lineweaver-Burk plots for Lipase AK and IM-AK-catalyzed glycerolysis of tuna oil.
Figure 1

Organic solvent

Content of MAG and PUFA (wt%)

1) acetone
2) acetone/isooctane (1:1 v/v)
3) acetone/isooctane (1:3 v/v)
4) acetone/isooctane (3:1 v/v)
5) acetone/hexane (1:1 v/v)
6) acetone/hexane (1:3 v/v)
7) acetone/hexane (3:1 v/v)
8) acetone/MTBE (1:1 v/v)
9) acetone/MTBE (1:3 v/v)
10) acetone/MTBE (3:1 v/v)
11) isooctane
12) hexane
13) MTBE
Figure 2

Content of MAG and PUFA (wt%)

% water

4 6 8 10 12

MAG PUFA

20.7 16.3 2.7 15.5 15.3
16.7 10.3 2.6 15.1 1.04

0 5 10 15 20 25
Figure 3

![Bar chart showing the content of MAG and PUFA at different fish oil percentages. The x-axis represents fish oil percentages (5% to 70%), and the y-axis represents content of MAG and PUFA (in wt%). The chart includes bars for MAG and PUFA at various fish oil levels, with specific values indicated for each bar.](chart.png)
Figure 4

Content of MAG and PUFA (wt%) vs. IM-AK (%)

- MAG
- PUFA
Figure 5
Figure 6