Efficient Synthesis of Stearidonic Acid Enriched Triacylglycerol from Ahiflower Seed Oil via a Two-Step Enzyme Reaction

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Abstract: Stearidonic acid (SDA) is a plant-based n-3 polyunsaturated fatty acid with multiple biological activities. The enrichment of SDA and synthesis of triacylglycerol (TAG) were carried out consecutively via two lipase-catalyzed reactions, hydrolysis, and esterification. First, SDA was enriched into a glyceride fraction from ahiflower seed oil by Candida rugosa lipase-catalyzed hydrolysis. Under the optimum conditions of 35°C, 0.1% lipase powder of Lipase OF, and 50% buffer solution (based on the weight of total substrate), SDA was enriched from 21.6 to 40.7 wt% in glyceride fraction. SDA-enriched TAG was then synthesized from the SDA-enriched glyceride and the SDA-enriched fatty acid via esterification using an in-house immobilized lipase as a biocatalyst. The SDA-enriched fatty acid was obtained from part of the SDA-enriched glyceride by saponification and the in-house immobilized lipase was prepared from Eversa® Transform 2.0 using Lewatit VP OC 1600 as a carrier. The optimum reaction conditions for the synthesis of TAG were a temperature of 50°C, an enzyme loading of 10%, and a vacuum of 10 mmHg. A maximum conversion to TAG of ca. 94% was achieved after 12 h under the optimum conditions.

Key words: ahiflower seed oil, Candida rugosa, Eversa® transform 2.0, stearidonic acid, triacylglycerol

1 Introduction
n-3 Polyunsaturated fatty acids (n-3 PUFAs) have multiple biological and positive therapeutic effects on human health such as reducing inflammatory disease and cancer risk and enhancing anti-aggregatory and vasodilatory proteinoid production [1, 2]. Fish oil is a major source of PUFAs and is widely distributed as an n-3 PUFAs dietary supplement. However, fish oil has two major drawbacks: an unpleasant odor from belching after ingestion, and low oxidative stability. Moreover, there is a study reported that high oxidative stability. Moreover, there is a study reported that the heavy metal toxins such as mercury can accumulate in fish oil. Mercury exposure has been a prolonged health issue in pregnant women and children because it can damage the nerves, kidneys, and liver [4-5].

On the other hand, flaxseed oil, which contains a high content of α-linolenic acid, is also widely distributed as an n-3 PUFAs dietary supplement. Advantageously, flaxseed oil does not have an unpleasant odor like fish oil, and it has high oxidative stability. Moreover, there is a study reported on cardiovascular disease in vegetarians, even though they have ingested only vegetable foods [6]. Thus, vegetarians prefer flaxseed oil over fish oil as an n-3 PUFAs dietary supplement. α-Linolenic acid is the most abundant n-3 PUFAs in the flaxseed oil; however, it requires additional bioconversion when it is absorbed into the body. Furthermore, α-linolenic acid is converted to stearidonic acid (SDA, C18:4, n-3), eicosapentaenoic acid (EPA, C20:5, n-3), and docosahexaenoic acid (DHA, C22:6, n-3). However, because of the presence of Δ6-desaturase as a limiting enzyme, the conversion rate of dietary α-linolenic acid to EPA and DHA is inefficient compared with that of SDA [7, 8]. Therefore, the level of interest in SDA is increasing because it provides EPA and DHA more efficiently. SDA, n-3 PUFAs, is known to be contained in several plant seed oils such as ahiflower seed oil, echium seed oil, and blackcurrant seed oil [9]. Recently, ahiflower seed oil has been marketed as a novel dietary supplement for n-3 PUFAs.

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Several attempts have been made to enrich n-3 PUFAs using enzymatic methods such as lipase-catalyzed selective hydrolysis or alcoholysis of marine oils\textsuperscript{10, 11}. The ethyl ester form of n-3 PUFA is currently enriched by molecular distillation in the industrial field. However, it has been reported that the triacylglycerol (TAG) form is absorbed easier in the human body than the fatty acids ethyl ester form. The fatty acid ethyl ester form performs incomplete absorption and is a poorer substrate for pancreatic lipase than TAG\textsuperscript{12}. Thus, several papers have reported on the synthesis of n-3 PUFA-enriched TAG via lipase-catalyzed esterification\textsuperscript{13, 14}.

The aim of this study is to synthesize SDA-enriched TAG from ahiflower seed oil via lipase-catalyzed reactions using an SDA-enriched glyceride instead of glycerol as a substrate. In most previous studies, the enrichment of n-3 PUFAs and the TAG synthesis have been performed separately. However, in the present study, SDA enrichment and TAG synthesis were carried out consecutively via two lipase-catalyzed reactions: selective hydrolysis, and esterification (Fig. 1). In the first step, SDA was enriched into glyceride obtained from ahiflower seed oil by Candida rugosa lipase-catalyzed hydrolysis and SDA-enriched glyceride was separated from the reaction mixture using molecular distillation. In the second step, SDA-enriched TAG was synthesized from the SDA-enriched glyceride and the fatty acid, obtained from part of the SDA-enriched glycerides by saponification via immobilized lipase-catalyzed esterification under vacuum conditions. Recently, in our previous studies\textsuperscript{15, 16}, various types of oleochemicals such as a plasticizer, a bio-lubricant, and an ingredient for cosmetics were effectively synthesized with a newly prepared immobilized lipase from Eversa\textsuperscript{®} Transform 2.0 (full commercial name: Eversa\textsuperscript{®} transform 2.0), which was purchased from Novozymes Korea (Seoul, Korea). The enzyme used for immobilization was a liquid Eversa lipase from Thermomyces lanuginosus, Lipozyme RM IM from Rhizomucor miehei, and Novozym 435 from Candida antarctica were purchased from Novozymes Korea (Seoul, Korea). The enzyme used for immobilization was a liquid Eversa lipase from Thermomyces lanuginosus (full commercial name: Eversa\textsuperscript{®} transform 2.0), which was purchased from Novozymes Korea (Seoul, Korea). Lewatit VP OC 1600, for use as a carrier, was purchased from Lanxess Energizing Chemistry (Obernburg, Germany). All other chemicals used in this study were purchased as an analytical grade unless otherwise specified.

2 Experimental

2.1 Materials

Ahiflower seed oil was donated by Natural Crop International (Kensington, Canada). Lipase OF from Candida rugosa was purchased from Meito Sangyo Co., Ltd. (Tokyo, Japan). Glycerol and falling-film distillation glassware (Catalog No. Z250236) were purchased from Sigma Aldrich Korea Co. (Seoul, Korea). Lipozyme TL IM from Thermomyces lanuginosus, Lipozyme RM IM from Rhizomucor miehei, and Novozym 435 from Candida antarctica were purchased from Novozymes Korea (Seoul, Korea). The enzyme used for immobilization was a liquid Eversa lipase from Thermomyces lanuginosus (full commercial name: Eversa\textsuperscript{®} transform 2.0), which was purchased from Novozymes Korea (Seoul, Korea). Lewatit VP OC 1600, for use as a carrier, was purchased from Lanxess Energizing Chemistry (Obernburg, Germany). All other chemicals used in this study were purchased as an analytical grade unless otherwise specified.

2.2 Candida rugosa lipase-catalyzed hydrolysis

Candida rugosa lipase-catalyzed hydrolysis was carried out according to the modified method of Tanaka et al.\textsuperscript{17}. Lipase-catalyzed hydrolysis of ahiflower seed oil was carried out in a flat-bottom glass vessel (internal diameter: 3.5 cm, height: 8 cm) equipped with a water jacket for temperature control. Ahiflower seed oil (1.5 g) and sodium phosphate buffer solution (1.5 g, 50 mM, pH 7.0) were placed in this vessel and heated to the desired temperature using a water circulator. The reaction was initiated by the addition of Lipase OF to the substrate mixture with stirring at 250 rpm. Samples (50 µL) were withdrawn periodically for thin layer chromatography (TLC) and gas chromatography (GC) analysis. The SDA-enriched glyceride was isolated from the reaction mixture obtained by lipase-catalyzed hydrolysis using the falling-film distillation system with a distillation head temperature of 197°C, the vacuum of 2 torr.
and a falling speed of 5 drops per min.

2.3 Enzyme immobilization

Enzyme immobilization was conducted according to a previously described method\(^{30}\). Lewatit VP OP 1600, for use as a hydrophobic carrier, was presoaked in absolute ethanol for 3 h to condition it so that the enzyme solution could physically enter the pores of the carrier in a later step. Ethanol in the carrier was then exchanged completely with sodium phosphate buffer (50 mM, pH 7.0). The enzyme solution was prepared by mixing liquid Eversa lipase (120 mL) with sodium phosphate buffer (30 mL). The enzyme solution (150 mL) was added to a flask containing the carrier (15 g). The mixture of enzyme solution and carrier was stirred on a water bath orbital shaker at 250 rpm and 30°C for 17 h. The carrier was then separated from the enzyme solution by filtration and immediately washed with buffer solution (150 mL) to remove unbound, or loosely bound lipase. The carrier with lipase was dried overnight at room temperature and then in a vacuum drying oven at 40°C for 12 h. The immobilized lipase was stored at 4°C prior to use. The quantity of protein in the enzyme solution before immobilization and the quantity of unbound protein in the enzyme solution after immobilization were determined according to the method by Lowry et al.\(^{30}\). The quantity of protein bound to the carrier (Lewatit VP OC 1600) was calculated using Eq. (1):

\[
\text{Protein in carrier (mg/g)} = \frac{(a-b)}{c} + \frac{(a-b) \times 10^{-3}}{(g)}
\]

where \(a\) is the quantity of protein in the enzyme solution before immobilization (mg), \(b\) is the quantity of unbound protein in the enzyme solution after immobilization (mg), and \(c\) is the quantity of carrier used for immobilization (g). The concentrations of protein in the enzyme solution before immobilization and after immobilization were 28.5 mg/mL and 10.7 mg/mL, respectively. Finally, the quantity of protein bound to the carrier (Lewatit VP OC 1600) was 137 mg per gram of the carrier.

2.4 Lipase-catalyzed esterification

Lipase-catalyzed esterification to synthesize SDA-enriched TAG was carried out in a 50-mL water-jacketed glass vessel as described in our previous study\(^{21}\). The composition of the reaction mixture obtained after molecular distillation was 7.2% fatty acid, 0.8% MAG, 37.9% DAG, and 54.1% TAG. From the composition of the glyceride obtained after molecular distillation, the number of moles of hydroxy groups in glyceride was calculated as the following Eq. (2):

\[
\text{Mole of hydroxy group in glyceride} = (a + 2b) - c
\]

Where \(a\), \(b\), and \(c\) are the number of moles of DAG in glyceride, the number of moles of MAG in glyceride, and the number of moles of fatty acid in glyceride.

The SDA-enriched glyceride (1.8 g, 0.71 mmole of hydroxy groups in glycerides) and SDA-enriched fatty acid (0.2 g, 0.71 mmole) were placed in the vessel, and the mixture was heated to the desired temperature using a water circulator. The SDA-enriched fatty acid was prepared from the SDA-enriched glyceride by saponification according to our previous study\(^{21}\). The reaction was initiated by adding lipase to the substrate mixture with stirring at 300 rpm under a defined vacuum. The vacuum was controlled using a micrometering valve (Swagelok, Solon, OH, USA) and monitored using a digital vacuum gauge (Teledyne, Thousand Oaks, CA, USA). Samples (50 mL) were withdrawn from the reaction mixture at appropriate time intervals and dissolved in chloroform. Individual samples were filtered through a 0.45-µm nylon microfilter ( Pall Corporation, Port Washington, NY, USA) to completely remove the enzymes.

2.5 Analysis of SDA content

The fatty acid and glyceride were separated by silica gel TLC using petroleum ether/diethyl ether/acetic acid (80:20:0.5, v/v/v), and detected under UV light using a solution of 2,7-dichlorofluorescein in methanol. The bands corresponding to glyceride (monoaoclylglycerol, diacylglycerol, and triacylglycerol) and fatty acid were scraped off the TLC plate and methylated according to the AOCS standard method Ce 2-66\(^{21}\). The fatty acid methyl ester was extracted with n-hexane (3 mL) and dried over sodium sulfate. A gas chromatograph (model 3800; Varian Inc., Palo Alto, CA, USA) equipped with a Supelcowax 10 fused-silica capillary column (30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA, USA) and flame ionization detector (FID) was used in the analysis. The column temperature was initially held at 180°C for 1 min, then increased to 230°C at a rate of 2°C/min, and finally held at 230°C for 20 min. The carrier gas was helium, and the total gas flow rate was 50 mL/min. The injector and detector temperatures were 240 and 250°C, respectively.

2.6 Positional distribution of fatty acids in ahiflower seed oil

Pancreatic hydrolysis was used to determine the positional distribution of fatty acid residues in the TAG from ahiflower seed oil. The oil (10 mg) was mixed with 2 mL of 1 M Tris-HCl buffer (pH 7.6), 0.5 mL of 0.05% bile salts, 0.2 mL of 2.2% CaCl\(_2\), and 3 mg of pancreatic lipase. The mixture was incubated in a water bath at 37°C for 2 min, vortexed vigorously, extracted with diethyl ether, and dried over anhydrous sodium sulfate. The mixture was then
placed on a silica gel TLC plate and developed with n-hexane/diethyl ether/acetic acid (50:50:1, v/v/v). The band corresponding to 2-MAG was scraped off, extracted with diethyl ether, methylated, and analyzed by GC. The fatty acid composition of the sn-1,3 position in the TAG was calculated from the sn-2 position and the total fatty acid composition in the TAG.

2.7 Measurement of conversion to TAG and degree of hydrolysis

The conversion to TAG and degree of hydrolysis were determined using gas chromatography (model 3800; Varian Inc., Palo Alto, CA, USA) equipped with a DB-1ht column (15 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA, USA) and flame ionization detector. The column temperature was held at 120°C for 3 min, increased to 370°C at a rate of 25°C/min, and then held at 370°C for 5 min. The carrier gas was helium at a flow rate of 1.5 mL/min and the split ratio was 1:50. The injector and detector temperatures were both set at 370°C. The conversion to TAG and degree of hydrolysis were calculated using Eqs. (3) and (4), respectively.

\[
\text{Degree of hydrolysis} = \frac{a}{a + b + c + d} \times 100 \quad (3)
\]

\[
\text{Conversion to TAG} (%) = \frac{d}{a + b + c + d} \times 100 \quad (4)
\]

where \(a\), \(b\), \(c\), and \(d\) are the weights of fatty acid, MAG, DAG, and TAG, respectively.

3 Results and Discussion

3.1 Enrichment of SDA via Candida rugosa lipase-catalyzed hydrolysis

It is well known that PUFAs such as \(\gamma\)-linolenic acid (GLA), EPA, and DHA are enriched efficiently via various lipase-catalyzed reactions\(^{23, 24}\). In this study, SDA was enriched in the glyceride fraction from ahiflower seed oil via Candida rugosa lipase-catalyzed hydrolysis. Two parameters, temperature and enzyme loading, were studied.

3.1.1 Temperature

The enzymatically catalyzed reaction is dependent on the temperature; therefore, the temperature is an essential parameter to determine the optimum condition for the enzymatic reaction. Enzyme activity is directly affected by temperature, and typically faster as temperature rises. However, excess heat may deactivate the enzyme and interfere with its selectivity\(^{25, 26}\).

Figure 2 depicts the effect of temperature on the enrichment of SDA from ahiflower seed oil via Candida rugosa lipase-catalyzed hydrolysis at the enzyme loading of 0.1% (based on the total weight of substrate), the weight ratio of 1:1 (oil to buffer solution), and the stirring speed of 300 rpm. The tested temperatures range was 25 to 45°C. A large increase in the degree of hydrolysis was observed as the temperature was increased from 25 to 35°C (Fig. 2A). By contrast, the difference in the degree of hydrolysis between reactions conducted at 35 and 45°C was negligible. The SDA content in the glyceride
at 25°C was drastically lower than those in the glyceride at 35, and 45°C throughout the entire reaction (Fig. 2B). However, the SDA content in the glyceride between 35 and 45°C was not significantly different. Most studies on the enrichment of PUFAs by selective hydrolysis using Candida rugosa lipase have been carried out at approximately 30 to 40°C [27]. Additionally, the supplier of this lipase recommends using a temperature of 40°C as an optimum as well. For the yield of SDA, similar yields were obtained after 120 min at 35 and 45°C, even though the yield of SDA at 35°C was higher than that at 45°C at the initial stage (Fig. 2C). Consequently, considering the effects on both the content and yield of SDA, 35°C was selected as the optimum temperature to enrich SDA from ahiflower seed oil using Candida rugosa lipase-catalyzed hydrolysis.

3.1.2 Enzyme loading

The amount of enzyme is important in an enzymatic reaction because it directly affects the activity, selectivity, and rate of reaction. The optimization of the enzyme loading maximizes the efficiency of the enzymatic reaction. Moreover, the use of excess enzyme should be avoided because of its adverse effects on economic viability.

Figure 3 depicts the effect of enzyme loading on the enrichment of SDA in ahiflower seed oil via Candida rugosa lipase-catalyzed hydrolysis at a temperature of 35°C, weight ratio of 1:1 (oil to buffer solution), and stirring speed of 300 rpm. The range of enzyme loading tested was between 0.05 and 0.2% (based on the total weight of the substrate). As the enzyme loading increased, the degree of hydrolysis increased steadily throughout the entire reaction period (Fig. 3A). During the first 60 min of the reaction, the SDA contents in the glyceride obtained with enzyme loadings of 0.1 and 0.2% were higher than that in the glyceride obtained with an enzyme loading of 0.05% (Fig. 3B). However, the SDA content between 0.1 and 0.2% was not significantly different. Meanwhile, the SDA content at an enzyme loading of 0.1% increased gradually up to ca. 40.2% as the reaction time increased from 60 to 120 min. However, no significant increases in the SDA content in the glyceride were observed when the reaction time was increased further. For the yield of SDA, a yield of ca. 75% was obtained at 120 min, which was the maximum SDA content (Fig. 3C).

The fatty acid compositions at the sn-1,3, and sn-2 positions in ahiflower seed oil and the glyceride obtained after Candida rugosa lipase-catalyzed hydrolysis are given in Table 1. There was no significant difference in the SDA content between sn-1,3 position and sn-2 position. However, the SDA content in the glyceride increased markedly after Candida rugosa lipase-catalyzed hydrolysis, and that of α-linolenic acid in the glyceride decreased significantly. It has been reported that Candida rugosa lipase exhibits strong selectivity toward Δ-9 fatty acids such as α-linolenic acid during hydrolysis or esterification [28, 29]. According to Baik et al. [30] Candida rugosa lipase exhibited a strong preference toward α-linolenic acid when esterification was carried out with fatty acid from echium oil and laurly alcohol. In another report, Rupani et al. [31] also reported that α-linolenic acid was enriched noticeably in the
fatty acid from 50% in the starting material to 72% with flaxseed oil via *Candida rugosa* lipase-catalyzed hydrolysis. Therefore, the results of the present study are consistent with these studies. In this study, SDA seems to be enriched because of the fatty acid specificity rather than the regiospecificity of *Candida rugosa* lipase. Hence, considering both the SDA content and yield, 0.1 g was selected as the optimum enzyme loading.

### 3.2 Synthesis of SDA-enriched TAG using an immobilized lipase

Numerous papers have been published on the synthesis of TAG from glycerol and fatty acids by lipase-catalyzed esterification\(^{32-34}\). However, most studies have employed Novozym 435 as an enzyme, which is one of the most expensive lipases. In the present study, SDA-enriched TAG was prepared using an in-house Eversa immobilized lipase as a biocatalyst, by esterification between the SDA-enriched glyceride and SDA-enriched fatty acid obtained by saponification from part of the SDA-enriched glyceride.

#### 3.2.1 Comparison of the esterification activities of immobilized lipases

The esterification activities of three commercial immobilized lipases (Novozym 435, Lipozyme RM IM, and Lipozyme TL IM), and the Eversa immobilized lipase for the synthesis of SDA-enriched TAG were investigated (Fig. 4). For these experiments, the temperature, the enzyme loading (based on the total weight of substrate), the molar ratio of the substrate, and the vacuum were kept constant at 60°C, 10%, stoichiometric ratio, and 5 torr. Among the three commercial lipases, Novozym 435 exhibited the highest activity for the synthesis of TAG. However, Novozym 435 and the Eversa immobilized lipase showed no significant difference in activity for the synthesis of TAG, even though the activity of Novozym 435 was slightly higher than that of the Eversa immobilized lipase after 4 h.

The results of the present study are consistent with these studies. In this study, SDA seems to be enriched because of the fatty acid specificity rather than the regiospecificity of *Candida rugosa* lipase. Hence, considering both the SDA content and yield, 0.1 g was selected as the optimum enzyme loading.

#### 3.2.2 Temperature

Temperature is a crucial factor in lipase-catalyzed esterification. Even if the same enzyme is used, the optimum temperature for lipase-catalyzed esterification depends on its source, the type of immobilization, substrate, and the type of reactor\(^{35, 37}\). The effect of temperature on the synthesis of SDA-enriched TAG via Eversa immobilized lipase-catalyzed esterification was studied as a function of the re-

### Table 1  Fatty acid compositions (wt%) at sn-1,3, and sn-2 positions of ahiflower seed oil and in the glyceride after *Candida rugosa* lipase-catalyzed hydrolysis\(^{a}\).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>TAG(^b)</th>
<th>Positions</th>
<th>SDA-enriched glyceride(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sn-2</td>
<td>sn-1,3</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (C16:0)</td>
<td>4.8 ± 0.1</td>
<td>1.9 ± 0.0</td>
<td>6.3 ± 0.0</td>
</tr>
<tr>
<td>Stearic acid (C18:0)</td>
<td>1.4 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>1.7 ± 0.0</td>
</tr>
<tr>
<td>Oleic acid (C18:1, Δ-9)</td>
<td>9.6 ± 0.0</td>
<td>16.7 ± 1.0</td>
<td>6.5 ± 0.5</td>
</tr>
<tr>
<td>Linoleic acid (C18:2, Δ-9)</td>
<td>10.8 ± 0.0</td>
<td>17.9 ± 0.9</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>γ-Linolenic acid (C18:3, Δ-6)</td>
<td>5.4 ± 0.2</td>
<td>8.9 ± 0.2</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>α-Linolenic acid (C18:3, Δ-9)</td>
<td>46.3 ± 0.4</td>
<td>31.2 ± 0.5</td>
<td>53.8 ± 0.3</td>
</tr>
<tr>
<td>Stearidonic acid (C18:4, Δ-6)</td>
<td>21.6 ± 0.6</td>
<td>22.7 ± 1.1</td>
<td>20.7 ± 0.6</td>
</tr>
</tbody>
</table>

\(^a\) Results are the average of triplicate determinations
\(^b\) Triacylglycerol of ahiflower seed oil
\(^c\) Glyceride after *Candida rugosa* lipase-catalyzed hydrolysis

*Fig. 4* Comparison of the esterification activities of immobilized lipases. Enzymes: A, Novozym 435; B, Lipozyme RM IM; C, Lipozyme TL IM; D, Eversa immobilized lipase. Reaction conditions: SDA-enriched glyceride from lipase-catalyzed hydrolysis (1.8 g), SDA-enriched fatty acid (0.2 g) obtained by saponification of the SDA-enriched glyceride, an enzyme loading of 10% (based on total weight of substrate), and a vacuum of 5 torr.
action time (Fig. 5). The range of temperature investigated was 20-60°C. For these experiments, the enzyme loading, the molar ratio, and the vacuum were kept 10% (based on the total weight of substrate), stoichiometric ratio, and 5 torr, respectively. During the first 6 h of reaction, both the initial reaction rate and the conversion to TAG substantially increased when the temperature was increased from 20 and 50°C. By contrast, when the temperature was increased from 50 to 60°C, the conversion to TAG in the first 6 h of the reaction did not change significantly. At both 50 and 60°C, TAG conversion increased steadily as the reaction time was increased from 6 to 12 h and then approached a plateau at longer reaction times. Overall, no significant differences were observed between 50 and 60°C for the TAG conversion throughout the reaction.

Hence, a temperature of 50°C was chosen as an optimum when the energy cost and enzyme stability were both considered. In our previous studies15, 16, the optimum temperature was also 50°C when lipase-catalyzed esterification for the synthesis of diisononyl adipate and neopentyl glycol diester were performed with the same immobilized lipase employed in this study.

3.2.3 Enzyme loading

The optimum enzyme loading is desired to synthesize TAG properly. An insufficient amount of enzyme will lead to an incomplete reaction, and an excess of the enzyme could adversely affect the reaction resulting in poor economic feasibility and rapid reaction with limited time allowance. Therefore, both the economic viability and product yield must be considered carefully when selecting the optimum enzyme loading. The effect of enzyme loading on the synthesis of SDA-enriched TAG via Eversa immobilized lipase-catalyzed esterification was investigated as a function of reaction time (Fig. 6). The range of enzyme loading investigated was 5-20%. For these experiments, the temperature, the molar ratio, and the vacuum were kept at 50°C, stoichiometric ratio, and 5 torr, respectively. The lowest conversion to TAG over the entire reaction was attained with an enzyme loading of 5%. A marked increase in conversion to TAG was observed as the enzyme loading was increased from 5 to 10%. However, there were no significant differences in the conversion to TAG when the enzyme loading was increased further. A maximum conversion of ca. 95% was achieved after 12 h with 10% enzyme loading. Hence, 10% was selected as the optimum enzyme loading for the subsequent vacuum experiment.

3.2.4 Vacuum

In the synthesis of SDA-enriched TAG via Eversa immobilized lipase-catalyzed esterification, the equilibrium position can be shifted to esterification if water is produced by the reaction of glyceride with fatty acid is removed effectively. On the other hand, the equilibrium position can be shifted to hydrolysis if the water produced by esterification is accumulated in the reaction mixture by inefficient removals. Meanwhile, an extra high vacuum can decrease the lipase activity because of the loss of essential water in lipase28. Thus, it is crucial to control the level of water in the reaction system during esterification for TAG synthesis. There are several methods to manage the water content in the reaction mixture such as nitrogen flushing, use of a saturated salt solution, molecular sieves, and vacuum29, 30.
In this study, a vacuum was employed to remove water produced by the lipase-catalyzed esterification. The effect of vacuum conditions on the synthesis of SDA-enriched TAG via Eversa immobilized lipase-catalyzed esterification was investigated as a function of the reaction time (Fig. 7). The range of pressure tested was between 1 and 200 mmHg. For these experiments, the temperature, the molar ratio, and the enzyme loading were kept at 50°C, stoichiometric ratio, and 10% (based on total weight of substrate), respectively. The level of vacuum greatly affected the TAG synthesis. The conversion to TAG increased significantly when the pressure was decreased from 200 to 50 mmHg. The conversion to TAG then increased slightly when the pressure was decreased from 50 to 10 mmHg through the entire reaction. When the pressure was decreased further to 1 mmHg, the conversion to TAG did not change significantly. The optimum vacuum level was a pressure of 5 mmHg or less in most studies on the synthesis of TAG using lipases. However, in this study, decreasing the pressure to values from 10 to 1 mmHg had no additional positive effect on the overall esterification results. Thus, 10 mmHg was selected as the optimum pressure.

The composition (wt %) of the reaction mixture obtained after 12 h of optimum conditions was 5.1% fatty acid, 0.8% DAG, and 94.1% TAG. The positional fatty acid compositions of SDA-enriched TAG synthesized by Eversa immobilized lipase-catalyzed esterification are given in Table 2. It was confirmed that SDA was evenly distributed along with the glycerol backbones of TAG synthesized using Eversa immobilized lipase. Thus, Eversa immobilized lipase did not exhibit regiospecificity in the synthesis of SDA-enriched TAG.

### 4 Conclusions

SDA-enriched TAG was effectively synthesized by two lipase-catalyzed reactions: hydrolysis for the enrichment of SDA, and esterification for the synthesis of TAG consecutively. In the lipase-catalyzed hydrolysis, SDA was enriched up to 40.7 wt% in the glyceride fraction from shiﬂower seed oil using Lipase OF from *Candida rugosa*. Next, SDA-enriched TAG was synthesized with the SDA-enriched glyceride instead of glycerol using in-house Eversa immobilized lipase. This study presents a novel strategy for the synthesis of TAG using the enzyme.

### Table 2  Positional fatty acid compositions (wt %) of SDA-enriched TAG synthesized by Eversa immobilized lipase-catalyzed esterification.

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<tr>
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<td>7.4 ± 0.9</td>
</tr>
<tr>
<td>Linoleic acid (C18:2, Δ-9)</td>
<td>7.5 ± 0.0</td>
<td>7.6 ± 0.5</td>
</tr>
<tr>
<td>γ-Linolenic acid (C18:3, Δ-6)</td>
<td>10.4 ± 0.1</td>
<td>11.6 ± 1.0</td>
</tr>
<tr>
<td>α-Linolenic acid (C18:3, Δ-9)</td>
<td>27.2 ± 0.3</td>
<td>26.4 ± 1.1</td>
</tr>
<tr>
<td>Stearidonic acid (C18:4, Δ-6)</td>
<td>41.6 ± 0.1</td>
<td>40.0 ± 2.0</td>
</tr>
</tbody>
</table>

a Results are the average of triplicate determinations

b SDA-enriched TAG
Synthesis of Stearidonic Acid Enriched Triacylglycerol from Ahiflower Seed Oil

Author Contributions
Changhwan Ju: Methodology, Validation, Formal analysis, Investigation, Writing- original draft. Yu Jin Lee: Methodology, Validation, Formal analysis, Investigation, Writing- original draft. Hui Su Yoon: Investigation, Formal analysis. Byung Hee Kim: Investigation, Writing-original draft. In-Hwan Kim: Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Project administration, Supervision, Funding acquisition.

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Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References


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