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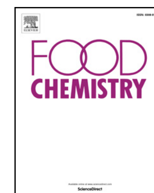

Production of stearidonic acid-rich triacylglycerol via a two-step enzymatic esterification

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ABSTRACT

The aim of this study was to synthesize stearidonic acid (SDA)-rich triacylglycerol (TAG) via a two-step lipase-catalyzed esterification under vacuum. SDA-rich fatty acid, which was prepared from echium oil via *Candida rugosa* lipase-catalyzed selective esterification, was used as the substrate. Two different immobilized lipases, Novozym 435 from *Candida antarctica* and Lipozyme TL IM from *Thermomyces lanuginosus*, were employed for the synthesis of SDA-rich TAG. In the first step, Novozym 435-catalyzed esterification of the SDA-rich fatty acid with glycerol was carried out for 2 h. In the second step, Lipozyme TL IM-catalyzed esterification of the reaction mixture from the first step was performed for an additional 10 h. The optimal reaction conditions for the second step were a temperature of 65 °C, an enzyme loading of 20%, and a vacuum of 0.7 kPa. Consequently, the maximum TAG conversion of ca. 86.4 wt% was obtained after 12 h via a two-step lipase-catalyzed esterification.

1. Introduction

The importance of polyunsaturated fatty acids (PUFA), especially omega-3 fatty acids, in human nutrition and disease prevention is scientifically recognized (Shahidi, & Wanasundara, 1998). Stearidonic acid (SDA) is a crucial *n*-3 PUFA and a metabolic intermediate in the conversion of α-linolenic acid (ALA) to highly unsaturated longer chain fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Guil-Guerrero, 2007; Whelan, 2009). SDA has similar biological properties as EPA and DHA, known as the most important fatty acids among the PUFA. In human body, ALA is able to be converted to SDA by Δ6-desaturase, and the SDA can be converted to EPA and DHA (Baik, No, Oh, & Kim, 2014). However, dietary ALA has a poor conversion rate to EPA and DHA because of the inefficiency of the Δ6-desaturase-catalyzed step (Guil-Guerrero, 2007; Surette, Edens, Chilton, & Tramposch, 2004). SDA can occur in blackcurrant seed oil and oils derived the *Boraginaceae* family (Petrik, McEntee, Johnson, Obukowicz, & Whelan, 2000). Of these oils, echium oil is one of SDA rich oils. In our previous studies (Baik, Kim, Oh, & Kim, 2015; Baik, No et al., 2014), SDA was successfully enriched from echium oil via a *Candida rugosa* lipase-catalyzed selective esterification.

It has been reported that triacylglycerol (TAG), which contains PUFA as its major fatty acids was more absorbed than ethyl esters containing PUFA in human body (Lawson, & Hughes, 1988). Moreover, resistance of TAG form on oxidation was also higher than that of the fatty acid form (Medina et al., 1999). There are number of papers on the synthesis of TAG via lipase-catalyzed esterification of fatty acid with glycerol using Novozym 435 from *Candida antarctica* as a biocatalyst (Cerdán, Medina, Giménez, González, & Grima, 1998; Hong, Kim, Yoon, Cho, & Kim, 2012; Medina et al., 1999). However, it is well known that Novozym 435 is one of the most expensive immobilized lipases currently available. In contrast, Lipozyme TL IM from *Thermomyces lanuginosus* is one of the cheapest lipases and has been widely employed for various esterification reactions (Reyes-Duarte et al., 2011; Xu, Du, Zeng, & Liu, 2004; Yamaguchi, Akoh, & Lai, 2004). However, it is impossible to synthesize TAG via Lipozyme TL IM-catalyzed esterification of fatty acids with glycerol because Lipozyme TL IM, which is prepared using silica gel as a carrier, aggregates in the presence of glycerol (Kim et al., 2017; Kristensen, Xu, & Mu, 2005; Yang, Rebsdorf, Engelrud, & Xu, 2005). To overcome this limitation of Lipozyme TL IM, TAG was synthesized with a strategy, which was to employ Novozym 435 and Lipozyme TL IM subsequently.

Thus, the goal of this study was to synthesize SDA-rich TAG via a two-step lipase-catalyzed esterification of SDA-rich fatty acid with

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glycerol using both Novozym 435 and Lipozyme TL IM as biocatalysts. The SDA-rich fatty acid was prepared from echium oil by *C. rugosa* lipase –catalyzed selective esterification according to our previous study (Baik, Kim et al., 2015; Baik, No et al., 2014. In this study, the first step reaction was carried out using Novozym 435 to investigate the optimal reaction time. Then, the reaction mixture from the first step was used as the substrate for the second step reaction, which was performed using Lipozyme TL IM. Several reaction parameters, including temperature, enzyme loading, and vacuum were investigated to optimize the second step reaction.

2. Materials and methods

2.1. Materials

Echium oil was obtained from De Wit Speciality Oils (Texel, Holland). Lipase OF (from *C. rugosa*) was obtained from Meito Sangyo Co., Ltd. (Nagoya, Japan). Novozym 435 (from *C. antarctica*), Lipozyme RM IM (from *Rhizomucor miehei*), and Lipozyme TL IM (from *T. lanuginosus*) were obtained from Novozymes (Seoul, Republic of Korea). Dodecanol (purity, ≥99.0%) and glycerol were obtained from Sigma Aldrich Co. (Seoul, Republic of Korea). Silica gel 60 for thin layer chromatography was obtained from Merck KGaA (Darmstadt, Germany). Other chemicals used were of analytical grade unless otherwise noted.

2.2. Preparation of fatty acid from echium oil

Echium oil (150 g) was added to 40% NaOH aqueous solution (150 mL) and 99% absolute ethanol (450 mL). The solution was then refluxed at 400 rpm with a magnetic stirrer for 1.5 h. Water (300 mL) was added to the saponified mixture and the aqueous layer including the saponifiable substance was acidified through adding 6 N hydrochloric acid and adjusted to pH 1 to convert the fatty acids. The upper layer including the fatty acid was extracted into *n*-hexane (300 mL) and washed twice with distilled water (150 mL). The *n*-hexane layer including the fatty acid was then dried with sodium sulfate anhydrous. Then *n*-hexane was removed with an evaporator at 35 °C. The remaining *n*-hexane in the fatty acid was eliminated by N_2 flushing at 40 °C.

2.3. Preparation of the SDA-rich fatty acid using C. rugosa lipase

The enzymatic esterification of the fatty acid from the echium oil with dodecanol was performed in a large scale reactor (1 L) using *C. rugosa* lipase in accordance with the method reported by Baik, No et al. (2014). An equimolar of the fatty acid (1.08 mol) and dodecanol (1.08 mol) were placed in a 1 L glass reactor and the glass reactor was heated to 30 °C using a water circulator. An initial portion of water (1.25 mL, 0.25 wt% of total substrate) was then loaded to the substrate. The esterification was then started by the addition of enzyme (10 g, 2 wt% of total substrate) and the substrate was stirred at 500 rpm with a magnetic stirrer. After 4 h, the SDA-rich fatty acid was isolated by the saponification of the reaction mixture containing the dodecanol, dodecyl esters, and fatty acids. The reaction mixture (100 g) was briefly mixed with *n*-hexane (1 L) and the mixed solution was filtered through sodium sulfate anhydrous to eliminate water and enzyme. 2% NaOH aqueous solution (200 mL) and 95% ethanol (200 mL) was added to the solution, and the mixture was placed into a separatory funnel. The lower layer was collected and placed into another separatory funnel and washed with *n*-hexane (1 L) to eliminate any residual dodecyl esters. The layer was subsequently acidified by adding concentrated hydrochloric acid (20 mL), and washed with *n*-hexane (200 mL) to recover the fatty acids. This fraction was subsequently washed three times using distilled water (20 mL) and filtered through sodium sulfate anhydrous. The *n*-hexane was then removed with an evaporator. The fatty acid composition of echium oil and SDA-rich fatty acid used in this study are shown in Table 1.

Table 1
Fatty acid composition (wt%) of echium oil and the SDA-rich fatty acid used as substrate.[a]

Fatty acids	Echium oil	SDA-rich fatty acid[b]
C16:0	7.4 ± 0.1	5.4 ± 0.4
C18:0	3.3 ± 0.1	6.6 ± 0.4
C18:1*n*9	14.8 ± 0.1	5.1 ± 0.2
C18:1*n*7	0.5 ± 0.0	0.6 ± 0.2
C18:2*n*6	14.9 ± 0.1	4.0 ± 0.1
C18:3*n*6	11.3 ± 0.1	31.9 ± 0.1
C18:3*n*3	33.4 ± 0.1	8.3 ± 0.1
C18:4*n*3	14.6 ± 0.0	38.1 ± 0.2

[a] Values represent the average of duplicate determination from different experimental experiments.
[b] The SDA-rich fatty acid was prepared by *Candida rugosa* lipase-catalyzed selective esterification in a large scale reactor.

2.4. The first step – Novozym 435-catalyzed esterification

For the first step reaction, glycerol (0.3 g; 3.2 mmole) and SDA-rich fatty acid (2.7 g; 9.7 mmole) were employed to synthesize TAG by lipase-catalyzed esterification under vacuum condition. The reactions were performed in a 50 mL glass reactor. The glass reactor was heated using a water circulator. To keep the initial water content of the reactants consistent, a vacuum was applied for 5 min at 0.1 kPa and 60 °C. Novozym 435 was loaded to a mixture of glycerol and SDA-rich fatty acid. The reaction was started with stirring at 300 rpm under a vacuum condition. The vacuum was regulated with a micro needle valve (Swagelok, Solon, OH, USA) and monitored by a vacuum gauge (Teledyne, Thousand Oaks, CA, USA). A sample (70 mg) was taken from the reaction mixture after stirring was stopped and the vacuum was released at appropriate intervals during the reaction. Individual samples were passed through a nylon microfilter (Pall Corporation, Port Washington, NY, USA) to completely eliminate the enzyme.

2.5. The second step – Lipozyme TL IM-catalyzed esterification

For the first step, Novozym 435 was employed for a short period and was then eliminated by a nylon microfilter (Pall Corporation, Port Washington, NY, USA). The first step reaction mixture consisted of fatty acids, monoacylglycerol (MAG), diacylglycerol (DAG), and TAG. For the second step, the esterification was performed with the first step reaction mixture using Lipozyme TL IM. The first step reaction mixture (3 g) was placed in a 50 mL glass reactor and the lipase was loaded. The reaction was started with stirring at 300 rpm under a vacuum condition. Samples (70 mg) were taken from the reaction mixture at defined intervals.

2.6. Analysis of products

To determine the conversion to TAG in the reaction mixture, samples (10 μL) corresponding to the various reaction times were dissolved in $CHCl_3$ (1 mL). The samples were then analyzed by a Varian 3800 gas chromatograph (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 a flame ionization detector (FID). The column was held at 125 °C for 2 min, and then heated to 365 °C at a rate of 18 °C min^{-1}. The column was then held at 365 °C for 5 min. Helium was used as a carrier gas with a flow rate of 1.3 mL min^{-1} and a split ratio of 1:40. The injector and detector temperatures were set at 370 °C.

The conversion to TAG was calculated by the following equation:

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