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A novel method for the synthesis of symmetrical triacylglycerols by enzymatic transesterification

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HIGHLIGHTS

- Symmetrical triacylglycerols (STAGs) had been synthesized by transesterification.
- Enzymatic transesterification for STAGs synthesis is a greener route.
- Enzymatic reaction using fatty acid vinyl ester as acyl donor is irreversible.
- Low acyl migration rate during transesterification were due to the irreversibility.

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ABSTRACT

A novel two-step enzymatic method is described in this study to synthesize symmetrical triacylglycerols (TAGs) with arachidonic acid (ARA) at the *sn*-2 position. The processes included the synthesis of 2-monoacylglycerols (2-MAGs) rich in 2-arachidonoylglycerol (2-AG) by enzymatic ethanolysis and the synthesis of symmetrical TAGs by enzymatic transesterification between 2-MAGs and vinyl palmitate. Under the optimal conditions, desired symmetrical TAGs were obtained at 89% yield. In this study, vinyl palmitate rather than palmitic acid was used as a novel acyl donor to react with 2-MAGs. It was the first study reporting the synthesis of symmetrical TAGs by enzymatic transesterification. The reaction using fatty acid vinyl ester as acyl donor is irreversible and temperature is low. Low-temperature reaction greatly suppressed the acyl migration of 2-MAGs and the irreversible reaction is much more effective compared to reversible reactions using free fatty acid and fatty acid ester as acyl donors.

1. Introduction

Polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA), have been shown to exhibit a variety of physiological functions in animal and human subjects (Lopez-Huertas, 2010; Harris et al., 2009; Farina et al., 2011). However, physiological functions of PUFAs are greatly affected by their occurring forms. It is well documented that PUFAs can be digested and enriched more effective in form of triacylglycerols (TAGs) as compared to those in form of free fatty acids and fatty acid esters (Neubronner et al., 2011; Dyerberg et al., 2010). Moreover, TAG structure also affects the physicochemical properties and biological activities of PUFAs due to the metabolism difference between *sn*-2 and *sn*-1,3 fatty acids of the TAGs (Mu and Porsgaard, 2005; Wijesundera et al., 2008; Zhang et al., 2009). A large body of evidence implies that symmetrical TAGs with PUFAs at the *sn*-2 position have advantages in clinical nutrition and treatment of disease (Wang et al., 2015; Osborn and Akoh, 2002). For these reasons, there is now great interest in the synthesis of symmetrical TAGs with PUFAs at the *sn*-2 position.

The importance of synthesizing TAGs with ARA at the *sn*-2 position is also associated with biological functions of 2-arachidonoylglycerol (2-AG), which is a current focus of considerable pharmaceutical interest to treat disorders such as obesity, chronic pain, anxiety and depression (Panikashvili et al., 2001; Katona and Freund, 2008; Holt et al., 2005; Di Marzo, 2008). Although some researchers have successfully synthesized pure 2-AG by chemical methods (Roche et al., 2012; Cartoni et al., 2004), 2-AG is extremely unstable. The ratio of 1-AG and 2-AG at equilibrium state is 9:1 (Andrews et al., 2008). Therefore, 2-AG is likely converted to 1-AG spontaneously at proper conditions during storage and usage, resulting in the loss of biological activities of 2-AG.

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The TAGs with ARA at the *sn*-2 position and 2-AG have the same metabolism route since *sn*-2 ARA at the TAGs is absorbed and enriched in form of 2-MAGs after being consumed due to the *sn*-1,3 specificity of lipases (Mu and Porsgaard, 2005; Small, 1991). Based on the metabolism pathway of TAGs, TAGs with ARA at the *sn*-2 position may have the same biological functions as compared to 2-AG. In contrast to 2-AG, TAGs stability is much higher. Thus, the synthesis of TAGs with ARA at the *sn*-2 position is also highly interest since such TAGs can exhibit not only biological functions of ARA itself, but also the activities of 2-AG. Differently from previous studies investigating synthetic methods of 2-AG, method of synthesizing symmetrical TAGs with ARA at the *sn*-2 will be studied in this study.

In the past five decades, only chemical (Stamatov and Stawinski, 2007; Vaique et al., 2010; Awl et al., 1989) and chemoenzymatic methods (Andrews et al., 2008; Haraldsson et al., 2000; Magnusson and Haraldsson, 2010) were reported for the synthesis of high regiopurity symmetrical TAGs with PUFAs at the *sn*-2 position in the literature. In these methods, many toxic solvents and reagents, such as 4-dimethylaminopyridine (DMAP), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDCI) and fatty acid chloride were used. Thus, these methods are not environmentally friendly. Compared to chemical and chemoenzymatic methods, lipase-catalyzed synthesis of symmetrical TAGs is greener. However, enzymatic methods, such as acidolysis, interesterification and esterification cannot result in the formation of high regiopurity TAGs (Zhang et al., 2009; Lee et al., 2010). The resulting TAGs by enzymatic methods are a mixture of TAG isomers and the desired TAG content in the crude mixture is usually 30–40% (Lee et al., 2010; Kawashima et al., 2001). In addition, these reported enzymatic methods for the synthesis of symmetrical TAGs usually need high reaction temperature (50–65 °C) and long reaction time (12–24 h).

In order to establish a highly efficient method for the synthesis of high regiopurity TAGs with ARA at the *sn*-2 position in a greener and sustainable manner, a two-step enzymatic transesterification was employed to synthesize the symmetrical TAGs. The processes included the synthesis of 2-monoacylglycerols (2-MAGs) by enzymatic ethanolysis and then the synthesis of the symmetrical TAGs by enzymatic transesterification. In this study, microbial oil, which is a safe and sustainable source of long chain polyunsaturated fatty acids (Wang et al., 2015), was used as a starting material for the synthesis 2-MAGs and symmetrical TAGs. To date, no study has reported the synthesis of symmetrical TAGs by enzymatic transesterification using fatty acid vinyl ester as acyl donor.

2. Methods

2.1. Materials

A fungal oil containing 48.8% ARA from *Mortierella alpina* ALK, was obtained from Guangzhou Rekon Edible Chemical Industry Co. Ltd. (Guangzhou, China). Immobilized lipase from *Candida antarctica* (Novozym 435) and Lipozyme RM IM (a commercial immobilized 1,3-specific lipase from *Rhizomucor miehei*) were kindly donated by Novozymes (Beijing, China). Vinyl palmitate (98%) was obtained from Tokyo Chemical Industry (Tokyo, Japan). Diolein (85% 1,3-diolein and 15% 1,2-diolein), 1-oleoylglycerol ($\geqslant$99%), 2-oleoylglycerol ($\geqslant$95%), methyl arachidonate and mixed fatty acid methyl ester standards were purchased from Sigma–Aldrich Chemical Co. Ltd. (Shanghai, China). Hexane and isopropanol used for HPLC analysis were chromatographically pure. All other organic solvents were purchased commercially and were of analytical grade.

2.2. Synthesis and purification of 2-AG

The enzymatic ethanolysis reaction was conducted with agitation at room temperature for 3 h by reacting 1 g of arachidonic-acid rich oil with 4 g of ethanol using 8% (w/w, relative to total reactants) Novozym 435 as catalyst. At the end of the reaction, the crude product was analyzed by HPLC after the lipase and solvent were removed.

The crude ethanolysis product was purified by solvent extraction to obtain purified 2-MAGs. Ethanolysis product obtained from 1 g of arachidonic-acid rich oil was dissolved in 15 mL of 85% (v/v) ethanol aqueous solution and then 10 mL of hexane was added. The mixture was poured into a separation funnel. After shaking, the ethanol aqueous phase containing 2-MAGs was collected and washed again with 10 mL of hexane twice. After solvent extraction, ethanol and water were evaporated under reduced pressure at 30 °C to obtain pure 2-MAGs.

2.3. Optimization of the synthesis of symmetrical TAGs with ARA at the sn-2 position by enzymatic transesterification

TAGs with ARA at the *sn*-2 were enzymatically synthesized by irreversible transesterification between synthetic 2-MAGs rich in 2-AG and vinyl palmitate. The design for the optimization experiments is listed in Table 1. The effects of the type of solvent, solvent amount, reaction temperature, lipase loading and reaction time were optimized as single factors based on the TAGs content in the crude mixture. All reactions were run in duplicate, and the results were reported as mean ± standard deviation.

2.3.1. Transesterification between 2-MAGs and vinyl palmitate

Optimization for symmetrical TAGs was carried out at controlled temperature by reacting 1 mmol of 2-MAGs and 2.5 mmol of vinyl palmitate. Subsequently, solvent was added into the reaction system to dissolve the reactants and Lipozyme RM IM lipase was used as catalyst to start the transesterification. For optimization of transesterification, the selected solvents included dichloromethane, hexane and acetone and solvent amounts were changed from 0 to 2 mL. The ranges of reaction temperature, lipase loading and reaction time were 0–70 °C, 6–22% (relative to total weight of reactants) and 30–210 min, respectively. At the end of the reaction, the lipase and solvent were removed and 1 mL hexane was used to dilute the product. The reaction product was quantified by HPLC as described in the following subsection.

2.4. The synthesis of symmetrical TAGs with ARA at the sn-2 position on a large scale

After the reaction conditions were optimized by single factor optimization, the synthesis of the TAGs was performed under the optimal conditions on a large scale. Namely, 2-MAGs (100 mmol) and vinyl palmitate (250 mmol) were mixed with agitation at 30 °C for 2.5 h in 50 mL hexane with 10% Lipozyme RM IM as catalyst. At the end of the reaction, the crude reaction product was quantified by HPLC after the removal of the lipase and solvent.

2.5. HPLC analysis of the enzymatic reaction product

For the separation and quantification of transesterification product including synthetic 2-AG and TAGs, HPLC-ELSD using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a LiChrospher Si column (250 mm × 4.6 mm, 5 μm particle size, Sigma–Aldrich Corp. K.K., Tokyo, Japan) was chosen and the products was eluted with a binary gradient of solvent A (100% of hexane) and solvent B (1:1:0.01 isopropanol/hexane/acetic acid, v/v/v) at 1.0 mL/min. Samples