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Improved enzymatic synthesis route for highly purified diacid 1,3-diacylglycerols

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ABSTRACT

The nutritional benefits and biological functions of diacylglycerols (DAGs) have attracted much attention regarding their synthesis. In this study, we improved the synthesis of diacid 1,3-DAGs by the enzymatic transesterification of 1-monoolein with a fatty acid vinyl ester as an acyl donor. First, 1-monoolein was prepared in 95% ethanol with Amberlyst resin as a catalyst by the cleavage of 1,2-acetonide-3-oleoylglycerol, which had been synthesized by enzymatic esterification of 1,2-acetonide glycerol with oleic acid. Second, purified 1-monoolein was reacted with vinyl palmitate in the presence of a lipase to obtain 1-oleoyl-3-palmitoylglycerol. Subsequently, the reaction conditions for the synthesis of diacid 1,3-DAGs were evaluated. Under the selected conditions, the crude mixture contained 90.6% pure 1-oleoyl-3-palmitoylglycerol. After purification by two-step crystallization, pure 1-oleoyl-3-palmitoylglycerol was obtained with a yield of 83.6%. The main innovations were the use of enzymatic transesterification to obtain highly purified diacid 1,3-DAGs instead of using chemical synthesis and the use of an irreversible reaction with a fatty acid vinyl ester as acyl donor rather than reversible reactions.

1. Introduction

Diacylglycerols (DAGs) are important amphiphilic emulsifiers and surfactants and are widely used in food, pharmaceutical and cosmetic industries [1,2]. In addition, 1,3-DAGs exhibit a variety of biological functions. For example, 1,3-DAGs can activate protein kinase C even though 1,2-DAGs produce better activation compared to 1,3-DAGs [3]. However, Dawson et al. [4] reported that 1,3-diolein produced similar activation of intracellular phospholipase A_2 compared to 1,2-diolein. Additionally, 1,3-diolein could increase phospholipase A_2 activity more than 1,3-didecanoin. Another study also found that 1,3-DAGs derived from oleic, linoleic and arachidonic acids were effective in activating Ca^{2+} and phospholipid-dependent protein kinase, whereas saturated 1,3-DAGs were much less effective [5]. These results indicate that fatty acid composition may influence the biological activities of 1,3-DAGs. Additional biological activities of 1,3-DAGs have been reported in other studies [3,6,7]. Although researchers have focused their studies on the biological functions of monoacid 1,3-DAGs, DAGs present in many biological membranes are composed of two fatty acids [8]. Thus, the synthesis of diacid 1,3-DAGs is also important due to the fatty acid composition of bio-membrane DAGs and the influence of DAG fatty acid composition on the biological activities.

In addition to the cellular activities of diacid 1,3-DAGs, the nutritional benefits of 1,3-DAGs also make its synthesis of interest. For example, Murase et al. [9] reported that α-linolenic acid-rich DAGs fed to rats inhibited fatty liver formation accompanied with an up-regulation of β-oxidation. A similar study conducted by Kim et al. [10] concluded that docosahexaenoic acid-rich DAGs improved hepatic steatosis and altered hepatic gene expressions in mice. Dietary DAGs are mainly composed of 1,3-DAGs due to the higher stability of 1,3-DAGs compared to 1,2-DAGs. Thus, the nutritional properties of 1,3-DAGs with different fatty acid compositions may differ.

To further develop DAG-based products for wide commercial applications, a detailed understanding of the physical properties of 1,3-DAGs, including mixed 1,3-DAGs and highly purified diacid 1,3-DAGs, is required [11–13]. Various pure monoacid 1,3-DAGs have been synthesized for the study of phase behavior [11]. However, the study of the physical properties of diacid 1,3-DAGs is limited, likely

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because these compounds are not commercially available, and the preparation of purified diacid 1,3-DAGs presents a challenge [11]. For these reasons, there has been great interest in the synthesis of highly purified diacid 1,3-DAGs.

To date, the method for the synthesis of diacid 1,3-DAGs has received little attention. In general, diacid 1,3-DAGs have been synthesized either by one-step enzymatic esterification [14,15] or chemical methods [11,13,16–18]. The one-step enzymatic esterification of glycerol with mixed fatty acids only produces approximately 15% diacid 1,3-DAGs due to the generation of monoacylglycerols (MAGs), monoacid 1,2-DAGs, diacid 1,2-DAGs, monoacid 1,3-DAGs and triacylglycerols (TAGs) and the difficult isolation of diacid 1,3-DAGs [14,15]. Therefore, the one-step enzymatic esterification is not feasible. In contrast, highly purified diacid 1,3-DAGs can be obtained by chemical synthesis. Generally, the chemical method for the synthesis of diacid 1,3-DAGs requires using toxic solvents, such as dichloromethane and triethylamine, and toxic catalysts, such as 4-dimethylaminopyridine (DMAP) and *N*,*N*-dicyclohexylcarbodiimide (DCC). Therefore, the synthesis of purified diacid 1,3-DAG by the chemical method is not environmentally sound.

To avoid the disadvantages of the previous methods, we have established a two-step enzymatic method for the synthesis of diacid 1,3-DAGs that included the use of lipase. First, 1-monolein was synthesized by enzymatic esterification and the cleavage reaction. Subsequently, enzymatic transesterification was conducted between synthetic 1-monoolein and vinyl palmitate using a lipase as catalyst to form 1-oleoyl-3-palmitoylglycerol. Compared to previous chemical synthesis, the two-step enzymatic reaction for the synthesis of diacid DAG utilizes less toxic reagents. Compared to the one-step enzymatic esterification, two-step enzymatic reaction is more effective.

2. Materials and methods

2.1. Materials

Palmitic acid vinyl ester (>96%) was purchased from Tokyo Chemical Industry (Shanghai, China). 1-Oleoylglycerol (≥99%) and diolein (85% 1,3-diolein and 15% 1,2-diolein) were obtained from Sigma–Aldrich Chemical Co. Ltd. (Shanghai, China). Novozym 435 (lipase B from *Candida antarctica*, immobilized on a macroporous acrylic resin) and Lipozyme RM IM (lipase from *Rhizomucor miehei*, immobilized on an anionic exchange resin) were obtained from Novozymes (Beijing, China). Novozym 435 and Lipozyme RM IM are immobilized lipases and have declared activities of 10,000 PLU (propyl laurate unit)/g and 275 IUN (inter-esterification units Novo)/g, respectively. All other reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China).

2.2. Enzymatic synthesis of 1-monoolein

1-Monoolein was synthesized by a two-step method based on our previous method with some modifications [19,20]. Briefly, 1,2-acetonide-3-oleoylglycerol was first synthesized. 1,2-Acetonide-3-oleoylglycerol was prepared in 25 mL hexane by reacting 50 mmol oleic acid with 60 mmol 1,2-acetonide glycerol at 65 °C for 12 h in the presence of 10% (w/w, relative to total reactants) Novozym 435 as a catalyst. At the end of the reaction, the crude product was used to synthesize 1-monoolein after the removal of lipase and solvent by filtration and by evaporation under reduced pressure, respectively.

The synthesis of 1-monoolein by the cleavage of unpurified 1,2-acetonide-3-oleoylglycerol (approximately 50 mmol) was conducted in 100 mL of 95% ethanol at room temperature for 24 h with 2 g Amberlyst-15 resin as catalyst. At the end of the reaction, the crude product was purified by recrystallization in hexane at −30 °C as described previously [19].

Table 1
Experimental design for optimization of enzymatic transesterification between 1-monoolein and palmitic acid vinyl ester.[a]

Level	X_1	X_2 (wt%)	X_3 (mL)	X_4 (°C)	X_5 (h)
1	Lipozyme RM IM	4	0.5	30	10
2	Novozym 435	6	1.0	35	1.5
3		8	1.5	40	2/0
4		10	2.0	45	2.5
5		12		50	3.0
6					4.0

[a] X_1 = the type of lipase; X_2 = lipase load; X_3 = solvent amount; X_4 = reaction temperature; X_5 = reaction time.

2.3. Enzymatic synthesis of 1-oleoyl-3-palmitoylglycerol

The design for the optimization experiments is presented in Table 1. The effects of the type of lipase, lipase load, amount of solvent, reaction temperature and duration on 1-oleoyl-3-palmitoylglycerol content in the crude reaction mixture were examined. When one factor was optimized, other factors were maintained at fixed values. After a factor optimization was completed, the selected value of this factor was used for subsequent factor optimizations. All reactions were performed in duplicate, and data were expressed as means ± standard deviation (SD).

The enzymatic transesterification of 1-monoolein with vinyl palmitate was conducted in hexane with agitation by reacting 2.1 mmol 1-monoolein with 2 mmol vinyl palmitate in 1 mL hexane with agitation at 35 °C for 2 h. Lipase was used as a catalyst to start the reaction. The effects of type of lipase (Novozym 435 and Lipozyme RM IM), lipase load (4, 6, 8, 10 and 12%), amount of solvent (0.5, 1.0, 1.5 and 2.0 mL), reaction temperature (30, 35, 40, 45 and 50 °C) and duration (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h) were investigated. At the end of the reaction, the lipase was removed by vacuum filtration, and the solvent was removed under reduced pressure. The crude reaction product was diluted to 0.8 mg/mL with hexane and subsequently quantified by HPLC, as described in the following section.

2.4. Purification of synthetic 1-oleoyl-3-palmitoylglycerol

After all factors were evaluated, the reaction was performed again under the selected conditions. At the end of the reaction, the resulting product was purified to separate 1-oleoyl-3-palmitoylglycerol. The main impurities in the crude reaction mixture were vinyl palmitate and 1-monoolein. 1-Oleoyl-3-palmitoylglycerol was separated from impurities based on their differing solubilities in different solvents.

First, diacid 1,3-DAG was dissolved in hexane (1:10, w/v) at 60 °C, and the mixture was maintained at 4 °C until completely crystallized. Thereafter, the crystal containing 1,3-DAG was collected, and the liquid phase containing vinyl palmitate was discarded. Second, semi-purified diacid 1,3-DAG was dissolved in methanol (1:10, w/v) at 60 °C, and the mixture was placed at 4 °C until completely crystallized. Subsequently, the crystal containing diacid 1,3-DAG was collected, and the methanol phase containing 1-monoolein was discarded. Finally, the fully purified 1-oleoyl-3-palmitoylglycerol was obtained after the removal of solvent under reduced pressure and quantified by HPLC.