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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.

2.5. GC analysis of 1,2-acetonide-3-oleoylglycerol and 1-monoolein

1,2-Acetonide-3-oleoylglycerolglycerol was quantified by GC without derivatization. Because the 1,2-acetonide glycerol came out together with the solvent, the purity of 1, 2-acetonide-3-oleoylglycerol was calculated based on the area ratio of the peak of 1,2-acetonide-3-oleoylglycerol and the total sample peaks, excluding 1,2-acetonide glycerol.

1-Monoolein was quantified by producing its ether derivative for GC determination. Pyridine (0.5 mL) was added followed by hexamethyldisilazane (0.15 mL) and trimethylchlorosilane (0.05 mL). The mixture was agitated for 15–30 s and allowed to stand for 10 min to allow the upper phase to clarify. The purity of 1-monoolein was calculated based on the peak area ratio.

Monoolein derivative and 1,2-acetonide-3-oleoylglycerol were quantified using GC-14B gas chromatography (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector (FID) using a 30 m × 0.25 mm × 0.25 μm (length × I.D. × film thickness) fused-silica capillary column PEG-20,000. The oven temperature was increased from 150 to 300 °C at a rate of 10 °C/min and then held at 300 °C for 10 min. The injector and detector temperatures were set at 320 °C. 1-Monoolein standard was used to identify the monoolein peak based on its retention time.

2.6. HPLC analysis of 1-oleoyl-3-palmitoylglycerol

The transesterification product before and after purification was analyzed by HPLC-ELSD, using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a LiChrospher Si column (25 cm × 0.46 cm, 5 μm particle size, Sigma–Aldrich Corp. K.K., Tokyo, Japan) at 35 °C and eluted with a binary gradient of solvent A (1:99 isopropanol/hexane, v/v) and solvent B (1:1:0.01, isopropanol/hexane/acetic acid, v/v/v) at 0.8 mL/min samples were diluted to 0.8 mg/mL and analyzed using the following gradient profile: solvent B was increased from 0 to 10% over 20 min. Finally, solvent B was held at 10% for 10 min. The total run time was 30 min.

Dioleins containing 85% 1,3-diolein and 15% 1,2-diolein were used as external standards, and the peaks were identified according to their HPLC retention times. The purity of the 1-oleoyl-3-palmitoylglycerol was calculated based on the peak area relative to the total peak area of a particular sample.

2.7. Statistical analysis

All data were analyzed by one-way ANOVA. Differences among the means were compared using Tukey's test with $P = 0.05$ set as the level of statistical significance.

3. Results and discussion

3.1. Enzymatic synthesis of 1-monoolein using acetonide as acyl acceptor

Under the selected reaction conditions, approximately 96.2% 1,2-acetonide-3-oleoylglycerol was produced in the crude reaction mixture during the enzymatic esterification between 1,2-acetonide glycerol and oleic acid. Subsequently, 1-monoolein was formed at a yield of 86.1% in the crude product after the cleavage of 1,2-acetonide-3-oleoylglycerol in 95% ethanol. After purification, pure 1-monoolein was obtained at a yield of 80.8%.

In general, 1-MAGs have been synthesized either by enzymatic esterification using glycerol as acyl acceptor [21] or by chemical methods [16,17,22,23]. The enzymatic esterification for the synthesis of 1-monoolein is usually performed by the esterification of oleic acid and glycerol. Under optimal conditions, the MAG content in the final product is low (approximately 50%) [8]. As a result, highly purified MAG has usually been synthesized by chemical methods. The chemical synthesis has its disadvantages, which include the use of toxic catalysts and solvents. Thus, the preparation of 1,2-acetonide-3-oleoylglycerol using lipase instead of DMAP offers advantages and has been used for the synthesis of lipid derivatives [24–26].

To avoid the disadvantages of the chemical methods and enzymatic esterification using glycerol as acceptor, the improved enzymatic synthesis using acetonide as acyl acceptor was used to prepare 1-monoolein. In the improved method, 1,2-acetonide-3-oleoylglycerol was synthesized first by the enzymatic esterification of 1,2-acetonide with oleic acid in hexane, followed by cleavage of the crude product in 95% ethanol to produce 1-monoolein. As a solvent, hexane is less toxic compared to dichloromethane used in our previous studies for the synthesis of 1-MAGs using acetonide as acyl acceptor. In addition, 1,2-acetonide-3-oleoylglycerol was cleaved in 100% methanol to produce 1-monoolein as described in our previous study. We found that methanolysis of 1,3-acetonide-3-oleoylglycerol occurred in methanol to form undesirable methyl oleate. This observation is consistent with other studies [20,27]. However, interestingly, the presence of a minor quantity of water inhibited the methanolysis side reaction. Based on this result, 95% ethanol was used as a solvent instead of pure methanol to conduct the cleavage of 1,2-acetonide-3-oleoylglycerol in this study. Therefore, the approach described herein for the synthesis of 1-monoolein is less toxic and more effective compared to previous studies [8,16]. Moreover, enzymatic synthesis using acetonide to obtain MAG has received much less attention compared to chemical methods. Only our group has reported the synthesis of 1-MAGs using acetonide as acyl acceptor by enzymatic esterification and cleavage reaction.

3.2. Enzymatic synthesis of 1-oleoyl-3-palmitoylglycerol by transesterification

The evaluation of the reaction conditions, including the type of lipase, lipase load, amount of solvent, reaction temperature and duration, aimed to obtain the maximum diacid 1,3-DAG content in the crude reaction mixture. HPLC is an effective technology to separate 1,3-DAGs from 1,2-DAGs based on the AOCS official method Cd 11b-96 (1999). However, HPLC cannot separate 1,3-DAGs with different fatty acid compositions. For example, HPLC cannot separate 1-oleoyl-3-palmitoylglycerol from 1-oleoyl-3-stearoylglycerol.

3.2.1. The effect of the type of lipase

The results regarding the effect of the type of lipase are presented in Fig. 1. Novozym 435 and Lipozyme RM IM were evaluated in this study. As shown in Fig. 1, using Lipozyme RM IM for the catalyst produced 82% diacid 1,3-DAG in the crude product compared with 80.6% with Novozym 435 as catalyst ($P > 0.05$). There was no significant difference in the content of diacid 1,3-DAGs even though the enzyme activities of Novozym 435 and Lipozyme RM IM were different [28]. Additionally, the cost of Novozym 435 is twice that of Lipozyme RM IM. Therefore, Lipozyme RM IM was selected as a catalyst for the synthesis of diacid 1,3-DAG.

Generally speaking, Novozym 435 is considered to be a non-specific lipase. However, the specificity of Novozym 435 is affected by solvent polarity [29,30] and the acyl donor [22,31]. When the esterification and ethanolysis reactions were conducted in polar solvents [28,32], Novozym 435 appeared to act as an *sn*-1,3 specific lipase rather than a non-specific lipase. In addition, Novozym 435 acts as an *sn*-1,3 specific lipase when used as catalyst during the transesterification of fatty acid vinyl ester with glycerol [31]. Thus, based on the previous results regarding the selectivity of Novozym 435, we hypothesized that Novozym 435 was a

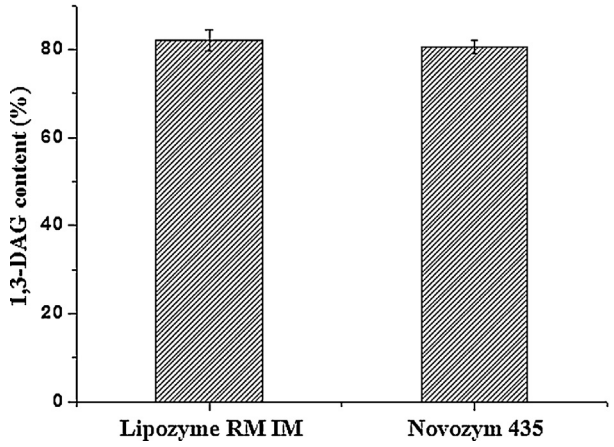

Fig. 1. Effect of the type of lipase. Reaction conditions: the reaction was performed with 10% lipase (w/w, relative to total reactants) as catalyst in 1 mL hexane with agitation at 35 °C for 2 h.

specific lipase toward to *sn*-1,3 positions when used for the enzymatic transesterification of 1-monoolein with palmitic acid vinyl ester. The results confirmed our hypothesis that Novozym 435 esterified vinyl palmitate to the *sn*-3(1) position of glycerol rather than the *sn*-2 position.

3.2.2. The effect of lipase load

When the substrates are saturated by the catalytic site of lipase, only a short time is needed to reach the maximum diacid 1,3-DAG content. Otherwise, the reaction will take a long time to reach the equilibrium state. Thus, the lipase load may affect the reaction time for reaching the equilibrium state. The results in Fig. 2 show that the lipase load had little effect on the diacid 1,3-DAG content in the crude product. No significantly difference was observed in the diacid 1,3-DAG content when the lipase load was increased from 4 to 12% ($P > 0.05$). The maximum content was observed with 12% lipase (Fig. 2), but addition of excess lipase is not economical. Therefore, 6% lipase was determined to be the optimal value for the synthesis of diacid 1,3-DAG to ensure completion of the reaction. For the industrial preparation of diacid 1,3-DAG, 4% lipase is more suitable due to the lack of a significant difference between 4 and 6% lipase load. Under these conditions, 82.5% 1-oleoyl-3-palmitoylglycerol was generated in the crude product.

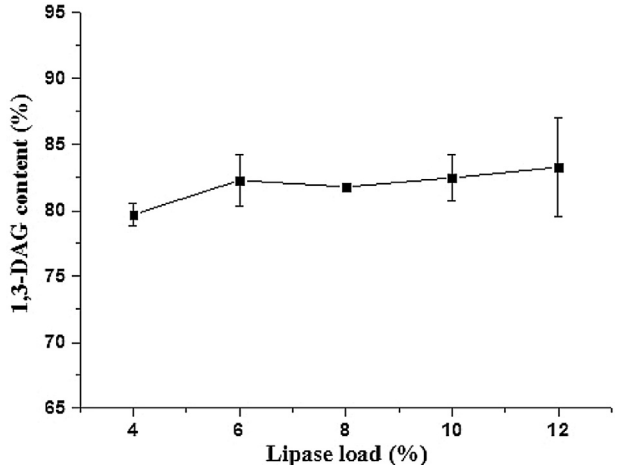

Fig. 2. Effect of lipase load. Reaction conditions: the reaction was performed in 1 mL hexane with agitation at 35 °C for 2 h with Lipozyme RM IM as catalyst.

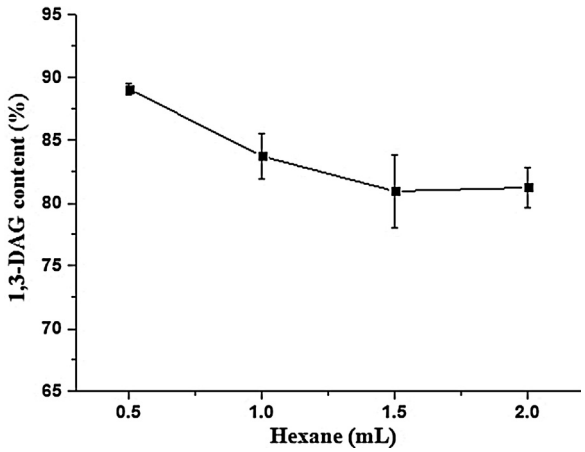

Fig. 3. Effect of the volume of solvent. Reaction conditions: the reaction was performed in hexane with agitation at 35 °C for 2 h using 6% Lipozyme RM IM as catalyst.

3.2.3. The effect of the amount of solvent

The amount of solvent may significantly affect the target product content quantity of reactants present in form of liquid in solvent and in form of solid will change when different quantity of solvent was added. The results regarding the effect of the amount of solvent are summarized in Fig. 3. The content of diacid 1,3-DAG tended to decrease with increasing volume of solvent from 0.5 to 1.5 mL. The content of diacid 1,3-DAG was maintained constant even though the volume of solvent was increased to 2 mL. Increasing the volume of solvent led to a decrease in the yield of diacid 1,3-DAG probably because the lipase and reactant concentrations were diluted. The decrease in the lipase concentration might lead to lower catalytic efficiency, whereas the reduction in the reactant concentration could lower the likelihood of molecular collision to form the diacid 1,3-DAG. Therefore, based on the results, 0.5 mL hexane was chosen for further reactions.

3.2.4. The effect of the reaction temperature

The reaction temperature is a key parameter for the lipase-catalyzed reaction. On the one hand, the reaction temperature affects the reaction rate and reaction time, causing reactions conducted at higher temperatures to result in higher reaction rates. On the other hand, the reaction temperature influences the lipase activity. A low reaction temperature may cause low lipase activity, whereas a high temperature may result in the inactivation of lipase. Thus, lipase-catalyzed reactions have an optimum reaction

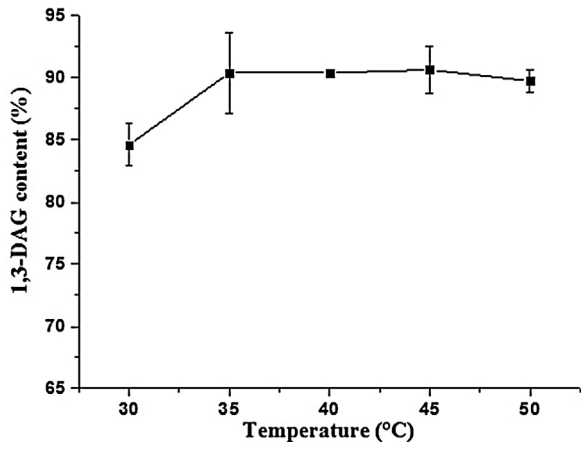

Fig. 4. Effect of the reaction temperature. Reaction conditions: the reaction was performed in 0.5 mL hexane with agitation at different temperatures for 2 h in the presence of 6% Lipozyme RM IM as catalyst.

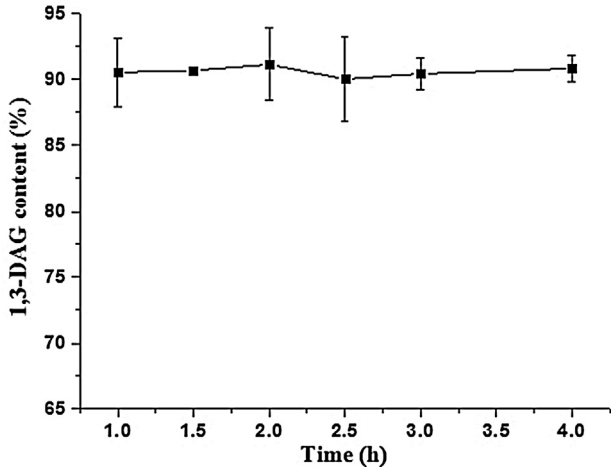

Fig. 5. Effect of the reaction time. Reaction conditions: the reaction was performed in 0.5 mL hexane with agitation at 35 °C using 6% Lipozyme RM IM as catalyst.

temperature. In addition, the reaction temperature also affects the acyl migration of partial acylglycerols. Usually, MAGs and DAGs are stable at temperatures below 40 °C, whereas the acyl migration rate increases with the increase in reaction temperature [32,33].

The results regarding the effect of the reaction temperature are shown in Fig. 4. The diacid 1,3-DAG content increased from 84.6 to 90.4% when the reaction temperature rose from 30 to 35 °C. Increasing the reaction temperature further did not affect the diacid 1,3-DAG content in the crude product. The low diacid 1,3-DAG content at 30 °C was due to the low solubility of diacid 1,3-DAG in hexane and the clouding of the solution at 30 °C after the 1,3-DAG was formed. However, when the reaction was performed at 35 °C, the reaction solution remained clear. Therefore, based on these observations, 35 °C was the temperature selected for further evaluations.

Normally, reactions between glycerol and an acyl donor such as free fatty acid, fatty acid methyl ester or triacylglycerol are reversible and must be conducted at 60–70 °C for an extended period of time [14,34]. However, when fatty acid vinyl ester is used as acyl donor, the reaction is irreversible [35] and can be performed at 0 °C [22,35]. Thus, fatty acid vinyl ester as an acyl donor is more effective than free fatty acid, fatty acid ester or TAG. Temperature is a very important parameter for the acyl migration of partial acylglycerols. Generally, reactions conducted at <40 °C will not cause the acyl migration of partial acylglycerols [36]. Thus, one of the advantages of using fatty acid vinyl ester as an acyl donor is the low temperature of the reaction, suggesting the prevention of acyl migration of 1,3-DAG to 1,2-DAG. In addition, the low reaction

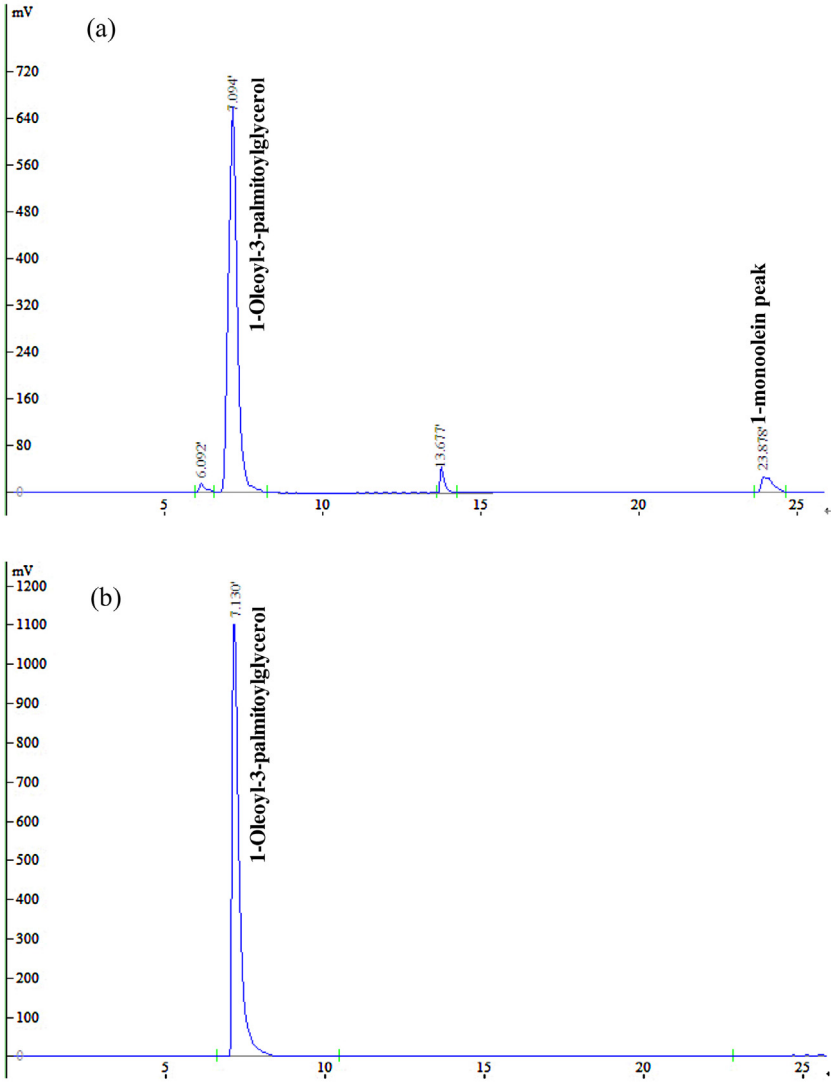

Fig. 6. GC chromatograms of synthetic 1-oleoyl-3-palmitoylglycerol before (a) and after purification (b).

temperature can reduce the loss of lipase activity and increase its reusability.

3.2.5. The effect of the reaction time

Finally, the effect of reaction duration was investigated. The results are outlined in Fig. 5. The reaction time did not influence the diacid 1,3-DAG content in the crude product when the reaction duration was increased from 1 to 4 h. The diacid 1,3-DAG content always remained constant. Therefore, 1 h was selected. Under these conditions, 90.6% diacid 1,3-DAG was produced (Fig. 6a).

Compared to previous studies of the synthesis of 1,3-DAG by the one-step enzymatic esterification of free fatty acid with glycerol [37], the reaction time is shortened significantly by enzymatic transesterification between fatty acid vinyl ester and 1-MAG. The short reaction time may also contribute to the irreversibility of the reaction. The decrease in reaction time could reduce the operating cost and acyl migration of 1,3-DAG. Thus, vinyl ester offers advantages over other acyl donors.

After the reaction, the crude product was purified by a two-step crystallization to remove impurities mainly consisting of vinyl palmitate and 1-monoolein. Because the polarity of 1-monoolein is the highest followed by 1-oleoyl-3-palmitoylglycerol and vinyl palmitate, 1-oleoyl-3-palmitoylglycerol can be separated from impurities based on the difference of solubility in different solvents. First, most of the diacid 1,3-DAGs and 1-monoolein crystallized out of the solution upon cooling of the crude product dissolved in hexane, whereas vinyl palmitate could be dissolved in hexane at very low temperature. Thus, at the first purification step, vinyl palmitate was separated from diacid 1,3-DAGs and 1-monoolein. Second, most of the diacid 1,3-DAG crystallized out of the solution upon cooling of the semi-purified product dissolved in methanol, whereas 1-monoolein remained soluble in methanol. Thus, after a two-step purification, the collected crystals were highly purified diacid 1,3-DAGs (Fig. 6b).

Studies on the synthesis of diacid 1,3-DAGs have been limited. Only the chemical method is available for the synthesis of purified diacid 1,3-DAGs, whereas one-step enzymatic esterification is much less effective. In our study, we improved the existing methods for diacid 1,3-DAGs synthesis through a two-step enzymatic reaction (esterification and transesterification). Thus, compared to the chemical method, our method is environmentally friendly. Compared to the one-step enzymatic esterification, our two-step enzymatic synthesis is more effective because the process avoids the formation monoacid glycerides. This new method is scalable due to the use of simple crystallization. Additionally, the transesterification reaction reaches equilibrium at 35 °C after 1 h. The reaction conditions are much milder compared with the one-step esterification that usually required reaction temperatures of 60–70 °C for 4–5 h [14]. Low reaction temperature is beneficial for preventing of acyl migration and increasing lipase reusability. The short reaction time and low reaction temperature may have contributed to the irreversibility of the reaction.

Conflict of interest

The authors declare no competing financial interest.

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