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ORIGINAL PAPER

Preparation of Diacylglycerol-Enriched Oil from Free Fatty Acids Using Lecitase Ultra-Catalyzed Esterification

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Abstract Production of diacylglycerol-enriched oil by esterification of free fatty acids (FFA) with glycerol (GLY) using phospholipase A1 (Lecitase Ultra) was investigated in this work. The variables including reaction time (2–10 h), water content (2–14 wt%, FFA and GLY mass), enzyme load (10–120 U/g, FFA and GLY mass), reaction temperature (30–70 °C) and mole ratio of GLY to FFA (0.5–2.5) were studied. The optimum conditions obtained were as follows: reaction temperature 40 °C, water content 8 wt%, reaction time 6 h, molar ratio of GLY to FFA 2.0, and an enzyme load of 80 U/g. Under these conditions, the esterification efficiency (EE) of free fatty acids was 74.8%. The compositions of the FFA and acylglycerols of the upper oil layer (crude diacylglycerol) of the reaction mixture were determined using a high temperature gas chromatograph (GC). The crude diacylglycerol from the selected conditions was molecularly distilled at 170 °C evaporator temperatures to produce a diacylglycerol-enrich oil (DEO) with a purity of 83.1% and a yield of 42.7%.

Keywords Diacylglycerol · Lecitase Ultra · Esterification · Free fatty acid · Molecular distillation

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Introduction

Diacylglycerols (DAG) are esters of glycerol (GLY) in which two of the hydroxyl group are esterified with free fatty acids (FFA). They present in two different isomeric forms which are 1,2 (2,3) DAG and 1,3 DAG. and is naturally present as a minor component of edible fats and oils of various sources [1]. DAG are important additives or carriers in the food, cosmetic, and pharmaceutical industries [2]. At present, DAG are regarded as healthful oils owing to their activity in preventing obesity [3]. Studies on animal models revealed that dietary of 1,3-DAG, as compared with triacylglycerol (TAG), significantly reduced the concentrations of serum and liver TAG [4]. This enriched composition of DAG (with a purity about 80 wt%) in DAG oil makes an important difference in their absorption and subsequent metabolism, particularly because of the 1,3-DAG. It has been suggested that the beneficial health effects of DAG are due to differences in digestion and absorption of TAG and DAG. However, The acyl migration from 1,2-DAG to 1,3-DAG observed in the rat stomach in presence of gastric acid indicated the same metabolites of these two isomers which proved that 1,2-DAG had the same function as 1,3-DAG [5]. Recommendations and guidelines on the intake of good fat and a balance of types of fats have recently been issued [6]. In the United States, DAG oil has received the status "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration [7]. The application of DAG-enriched oil (DEO) is being marketed as a functional cooking oil in Japan and in United States [8].

Various methods are available for the preparation of DEO. Conventionally, DEO can be prepared by chemical glycerolysis of edible fat and oils with GLY at high temperatures (220 to 260 °C) in the presence of inorganic

alkalis such as sodium, potassium, and calcium hydroxides as catalysts [9]. The chemical synthesis under high temperature is not preferred since colored and odoriferous polymers are formed during the reaction [2]. DEO can be produced enzymatically through esterification, glycerolysis and partial hydrolysis processes [9–11].

Phospholipases A1 are of particular interest for industrial applications, to produce 2-acyl-lysophospholipids (emulsifier) and to degum edible oils by cleaving *sn*-1 acyl group of phospholipids [12]. Phospholipases have been described to undergo interfacial activation [13], similar to lipases [14, 15]. Recently, a new enzyme possessing phospholipase A1 activity, Lecitase Ultra, was patented [16] and made commercially available. Mishra et al. [17] observed that the commercial preparation of Lecitase Ultra consisted mainly of a single protein with a molecular mass of 35 kDa which displayed both phospholipase and lipase activities. The enzyme is obtained from a microorganism with a fusion of the genes of the lipase from *Thermomyces lanuginosa* and the phospholipase from *Fusarium oxysporum* [18]. This new enzyme combines the stability of the *T. lanuginosa* lipase enzyme and the activity of the *F. oxysporum* enzyme. Lecitase Ultra is a carboxylic ester hydrolase which displays activity towards both phospholipids and TAGs [19].

In a previous work, we reported partial hydrolysis of soybean oil using Lecitase Ultra to produce DEO [20]. However, about 30 wt% of FFA was produced as a byproduct of the process. To maximize the utilization of the feedstock, the byproduct from the partial hydrolysis process was esterified with GLY to produce DEO catalyzed by the enzyme. This new esterification process provided an approach of production of DEO by utilization of the released fatty acid from partial hydrolysis of soybean oil. Five reaction variables, including, reaction temperature, reaction time, water content, enzyme load, mole ratio of GLY to FFA were investigated to obtain the optimal conditions for esterification. Molecular distillation (MD) was employed to purify the DAG from the reaction mixture to produce the final DEO.

Materials and Methods

Materials

Refined soybean oil was purchased from Kerry Cereal and Oil Co., Ltd. (Shenzhen, Guangdong, China). Commercial phospholipase A1 (Lecitase Ultra) was obtained from Novozymes A/S (Bagsvaerd, Denmark). Lecitase Ultra enzyme activity was reported as 10,000 U/mL for acyl group hydrolysis. Standard fatty acid and partial glycerides (oleic acid, 1-monooleoyl-glycerol (MAG) standard, 1,3-dioleoyl-glycerol (DAG) standard, and trioleoyl-glycerol (TAG)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of FFA

FFA were prepared by partial hydrolysis of soybean oil using Lecitase Ultra followed by a purification process using MD, as described below. Five hundred grams of soybean oil were hydrolyzed by phospholipase A1 (Lecitase Ultra) at a reaction temperature of 45 °C, a water amount of 40 wt% (soybean oil mass), an enzyme load of 20 U/g (FFA and GLY mass), and a reaction time of 10 h. After the reaction was completed, the mixture was allowed to settle in a separatory funnel for 60 min, to separate it into two layers, the non-polar upper layer (crude diacylglycerol) and the lower aqueous layer. The crude diacylglycerol was molecularly distilled at 140 °C to obtain the released FFA [20]. The preparation was performed five times to obtain sufficient FFA.

Esterification of FFA with GLY

The esterification reaction was involved five variables with their ranges specified as: reaction time (2–10 h), water content (2–14 wt%, FFA and GLY mass), enzyme load (10–120 U/g, FFA and GLY mass), reaction temperature (30–70 °C) and mole ratio of GLY to FFA (0.5–2.5).

Each of FFA sample (14.0 g) was added to a 100-ml jacketed glass reactor which was heated by circulating water from a water bath, and GLY, deionized water and the enzyme was introduced. The reaction was conducted under vacuum pressure (5 kPa) using a water ring pump connected with a neck of the reactor. A magnetic impeller with a speed of 300 rpm was used to stir the reaction mixture.

After the reaction was completed, the reaction mixture was transferred to a separatory funnel to settle for 60 min into two layers. The upper oil layer, composed of unreacted FFA and acylglycerols, which was defined as crude diacylglycerol. The lower polar layer, composed of unreacted glycerol, and enzyme was not used in further reactions. The esterification efficiency (EE) of FFA to acylglycerols was calculated by the following equation:

$$\mathrm{EE}\ (\%) = \left(1 - \frac{\mathrm{AV_{OL}}}{\mathrm{AV_{DEO}}}\right) \times 100\% \qquad (1)$$

where $\mathrm{AV_{OL}}$ and $\mathrm{AV_{FFA}}$ refer to the acid value of the oil layer after esterification and the acid value of the FFA, respectively.

Reaction Process of the Esterification Under the Optimal Conditions

The reaction process of the esterification under the optimal conditions was conducted in a 1,000-ml jacketed glass reactor which was heated by circulating heat fluid from an oil bath. FFA (200.0 g) and GLY, deionized water and the enzyme were introduced. The reaction was conducted under reduced pressure (5 kPa) using a water ring pump connected with a neck of the reactor. A mechanical impeller with two plastic paddles of 3.0 cm radius moving at 200 rpm was used to stir the reaction mixture. An aliquot of about 5 mL of the reaction mixture was withdrawn at periodic intervals. The reaction mixture was centrifuged ($1{,}120\times g$) into two layers, and then the crude diacylglycerol was subject to GC analysis for the composition of acylglycerols.

Preparation of DEO by MD

The crude DAG from the esterification reaction consisted of unreacted FFA, monoacylglycerol (MAG), DAG and TAG. The DAG was purified by a MD80 molecular distillation equipment, with a falling film evaporator (area: 0.1 m^2) and an internal condenser (area: 0.05 m^2) (Guangzhou Hanwei Co., Ltd., Guangzhou, China). A jacketed glass vessel with a flow regulation valve was used to feed. The distillate and the residue were discharged to glass flasks. The vacuum system was composed of a mechanical pump and a diffusion pump. Heating of the evaporator was provided by a jacket circulated with heated oil from an oil bath and the roller wiper speed inside the evaporator was fixed at 300 rpm for operation.

The removal of FFA and MAG from the crude diacylglycerol (35.0 g for each) was performed at the various temperatures at a constant feeding rate of 40 mL/h. The distillate (composed of FFA and MAG) and the residual (composed of DAG and TAG) were collected by glass flasks. Other conditions for MD were listed as followings: evaporator vacuum = 0.5–1.0 Pa, condenser temperature = 50 °C and feeding temperature = 80 °C. The residue from the MD was defined as DEO. The yield of the DEO and the removal efficiency of FFA were calculated using the following equations:

$$\text{Yield of the DEO } (\text{wt}-\%) = \frac{m_{\text{DEO}}}{m_{\text{CD}}} \times 100\% \quad (2)$$

where m_{DEO} and m_{CD} were the mass of the DEO and the mass of the crude diacylglycerol, respectively.

$$\text{Removal efficiency } (\%) = \left(1 - \frac{\text{AV}_{\text{DEO}}}{\text{AV}_{\text{CD}}} \times \text{Y}_{\text{DEO}}\right) \times 100\% \quad (3)$$

where AV_{DEO} and AV_{CD} were the acid value of the DEO and the acid value of the crude diacylglycerol; and Y_{DEO} was the yield of the DEO, respectively.

Analysis for Acylglycerols by High Temperature GC

The composition of acylglycerols in the samples was analyzed using an Agilent 7820A GC equipped with a capillary column (DB-1ht, 15 m × 0.25 mm × 0.10 μm, Agilent Technologies Inc., Palo Alto, CA, USA) and a flame ionization detector (FID).

Nitrogen was used as the carrier gas at a flow rate of 4.41 mL/min. The oven temperature program was: initial temperature at 100 °C increased to 220 °C at 50 °C/min, increased to 290 °C at 30 °C/min, then increased to 330 °C at 50 °C/min, then increased to 380 °C at 50 °C/min and holding for 3 min. The injection was performed in split mode with a split ratio of 20:1. The injector and detector temperatures were set at 380 and 400 °C, respectively. Samples were dissolved in hexane at a concentration of 10.0 mg/mL, and 2.0 μL of sample was injected onto the column. Levels of FFA, monoacylglycerols, diacylglycerols and triacylglycerols in the oils were quantified using calibration curves. All the determinations were performed in duplicate, and the means were reported.

DAG Fraction Analysis

The *sn*-1,3-DAG and *sn*-1,2 (2,3)-DAG components of the DEO were isolated by silica gel plates (SIL GF254, 20 × 20 cm, 0.25 mm), using benzene/diethyl ether/acetic ether/acetic acid (80:10:10:0.2, vol/vol) as the mobile phase. The *sn*-1,3-DAG fraction (RF = 0.87) and the *sn*-1,2 (2,3)-DAG fraction (RF = 0.78) were scraped off after visualization by iodine vapor and extracted with diethyl ether (2 mL ×3). The diethyl ether was removed with a evaporator, and the remainders were dissolved in 0.50 mL. Then the samples were analyzed with the method mentioned above: analysis for acylglycerols by high temperature GC. The ratio of *sn*-1,3-DAG and *sn*-1,2 (2,3)-DAG was calculated using peak area data. All the determinations were performed in duplicate, and the means were reported.

FFA Composition Analysis

The FFA compositions of the free fatty acid which was prepared by partial hydrolysis of soybean oil and the DEO were analyzed by GC (Agilent 7820A) equipped with a capillary column (DB-1ht, 15 m × 0.25 mm × 0.10 μm, Agilent Technologies Inc., Palo Alto, CA, USA and a flame ionization detector (FID). The derivatization of FFA

and DEO before injection were described as follows: samples of 4.0 g DEO were dissolved in 40.0 mL of methanol and then 0.5 mL of 1.0 M methanolic KOH was added, after 10 min reaction at the boiling point of methanol, *n*-hexane (20 mL) and water (40 mL) were added, and then the mixture was transferred to a separatory funnel. The upper organic phase was dried over anhydrous Na_2SO_4, and then concentrated under a nitrogen steam for analysis. A 4.0 g sample of FFA from the partial hydrolysis was esterified with 50.0 mL of 1.0 M methanolic H_2SO_4 for 15 min at the boiling point of methanol, and then partitioned between *n*-hexane (30 mL) and water (100 mL) in a separatory funnel. The upper organic phase was dried and concentrated for analysis.

The injection was performed in split mode with a split ratio of 20:1. Nitrogen was used as the carrier gas at a flow rate of 4.41 mL/min. The oven temperature program was: initial temperature at 140 °C, increased to 230 °C at 6 °C/min. The injector and detector temperatures were both set at 240 °C. Samples were dissolved in hexane at a concentration of 10.0 mg/mL, and 2.0 μL of the sample was injected onto the column. The FFA composition reported was based on the area response using an FID. All the determinations were performed in duplicate, and the means were reported.

Acid Value

Acid values of the samples were determined according to AOCS Method Cd 3d-63 by titration of FFA with 0.1 M KOH solution. All the determinations were performed in duplicate, and the means were reported.

Results and Discussion

Esterification of FFA with GLY

Theoretically, for an esterification reaction, a reverse reaction of hydrolysis, water should be avoided in the reaction to obtain a high EE. However, in an enzyme-catalyzed esterification, a small amount of water in the initial stage of the reaction is necessary to shift the enzyme to its active conformation and to achieve a high catalytic activity [21].

Thus it is necessary to add some water to the reactants to activate the Lecitase Ultra but to remove water generated to accelerate the reaction rate in a positive direction during the reaction. In this study, the EEs were 30.53 and 8.28%, under reduced pressure (5 kPa) and normal pressure, respectively. Other same parameters for these two reactions carried out at different pressures were: a reaction time of 6 h, an enzyme load of 20 U/g, a water content of 8 wt% (FFA and GLY mass), a reaction temperature of 50 °C and a mole ratio of GLY to FFA of 1:2. Lo et al. [4] studied the the yield and the productivity of DAG produced by the immobilized enzyme-catalyzed esterification reaction of FFA with GLY and it was strongly dependent on the vacuum conditions.

The effect of water content from 2.0 to 14.0 wt% on the EE of FFA is shown in Fig. 1a. The EE of FFA increased slowly at low water content and reached a maximum of 47.5% at a water content of 8.0 wt%, an enzyme load of 60 U/g (FFA and GLY mass), a reaction time of 4 h, a reaction temperature of 50 °C, and a mole ratio of GLY to FFA of 1:2. However, the EE decreased with the increase in water content at over 8.0 wt%, and reached a minimum of 17.07% at a water content of 14.0 wt%. Thus a critical water amount could be added to activate the enzyme to its optimal activity in this initial stage, and to be efficiently removed without inhibition of the esterification.

The effect of reaction temperature on the EE of FFA is depicted in Fig. 1b. Increasing the temperature increased the EE of FFA and it reached a maximum of 57.2% at a reaction temperature of 40 °C, an enzyme load of 60 U/g (FFA and GLY mass), a reaction time of 4 h, and a water content of 8 wt% (FFA and GLY mass), molar ratio of GLY to FFA of 1:2. Further increases in temperatur to over 60 °C resulted in a marked decrease in the EE. The optimal temperature for esterification using Lecitase Ultra is consistent to that of our previous study in a partial hydrolysis reaction using the same enzyme [10].

The mole ratio of GLY to FFA was very critical to the composition of final product as well as the EE of esterification reaction. The EE of FFA increased with increase of mole ratio of GLY to FFA and reached the maximum of around 80.3% when the GLY to FFA molar ratio was 2.0 (see Fig. 1c). However, The EE decreased markedly with the increase of ratio from 2.0 and 3.0. Too much GLY increased the viscosity of the reaction system as well as the difficulty of removal of water formed in the reaction which was negative to the mass transfer and reaction rate of the reaction, respectively.

The effects of reaction time and enzyme load on the EE of FFA are shown in Fig. 1d, e, respectively. Upon 6 h, an apparent equilibrium was attained. The EE increased rapidly at low levels but became flat at high levels. The esterification of GLY and FFA was a interface reaction since GLY was immiscible with FFA. At a low enzyme load, Lecitase Ultra adsorbed at the GLY/FFA interface was below the saturation level, so the reaction rate was dependent upon enzyme load. After the interface was saturated with enzyme at a high load, increasing the enzyme load resulted in no significant increase in the reaction rate of esterification. A similar result was observed when lipase was used to hydrolyze palm oil in an interface reaction

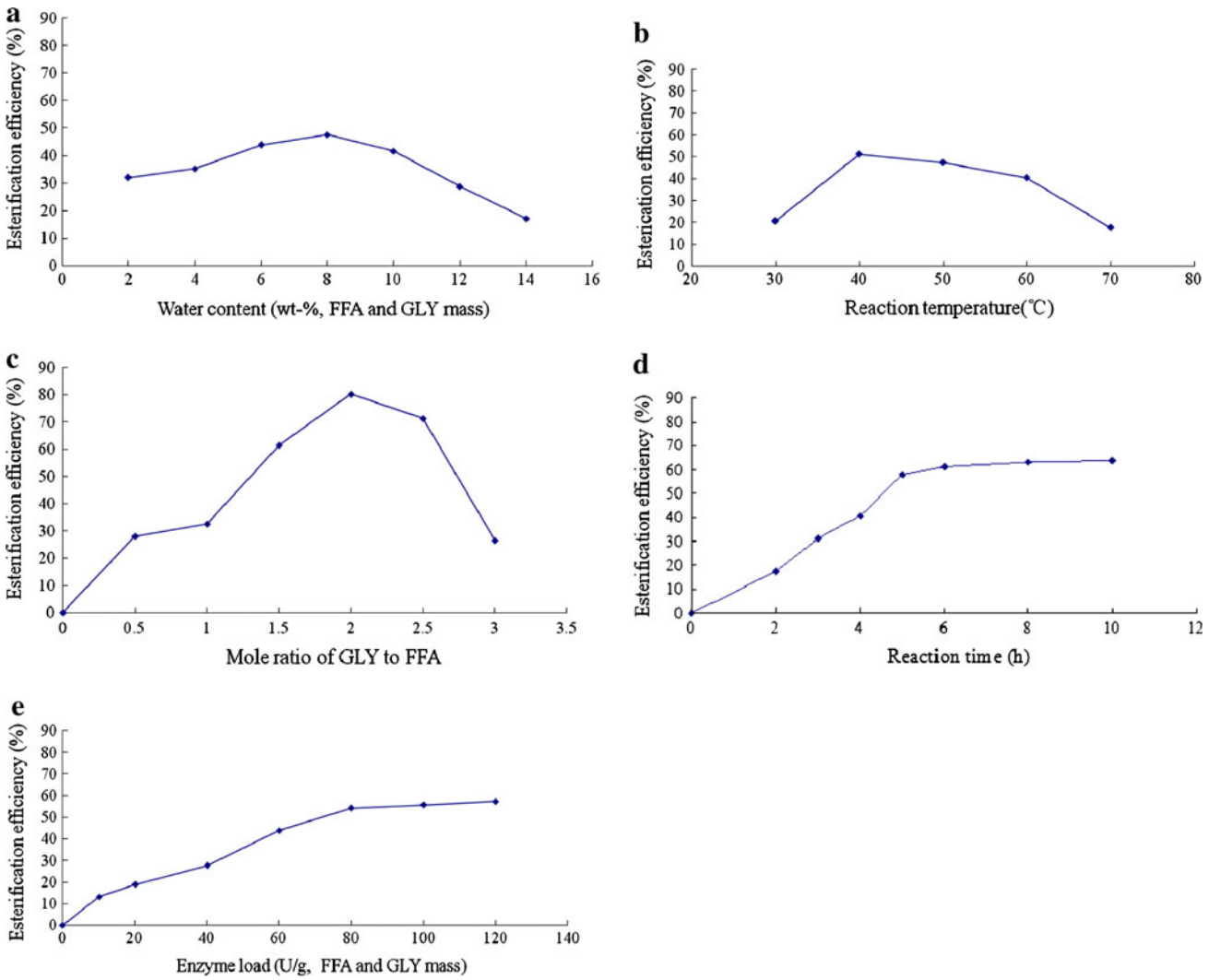

Fig. 1 Effects of variables on the esterification efficiency of free fatty acid (FFA) with glycerol (GLY) using Lecitase Ultra. **a** Water content; at an enzyme load of 60 U/g (FFA and GLY mass), a reaction time of 4 h, a reaction temperature of 50 °C, and a mole ratio of GLY to FFA of 1:2. **b** Reaction temperature; at an enzyme load of 60 U/g (FFA and GLY mass), a reaction time of 4 h, a water content of 8 wt% (FFA and GLY mass), and a mole ratio of GLY to FFA of 1:2. **c** Mole ratio of GLY to FFA; at an enzyme load of 60 U/g (FFA and GLY mass), a reaction time of 4 h, a water content of 8 wt% (FFA and GLY mass), and a reaction temperature of 40 °C. **d** Reaction time; at an enzyme load of 60 U/g (FFA and GLY mass), a water content of 8 wt% (FFA and GLY mass), a reaction temperature of 40 °C, and a mole ratio of GLY to FFA of 2:1. **e** Enzyme load; at a reaction time of 6 h, a water content of 8 wt% (FFA and GLY mass), a reaction temperature of 40 °C, and a mole ratio of GLY to FFA of 2:1. *FFA* free fatty acid, and *GLY* glycerol

system [22]. The reaction time of 6 h and enzyme load of 80 U/g were chosen economically as the selected reactions since no remarkable increases were observed at higher levels.

In this process, the best reaction conditions for catalysis from the results were: reaction temperature 40 °C, water content 8 wt% (GLY and FFA mass), reaction time 6 h, mole ratio 2.0 of GLY and an enzyme load of 80 U/g (GLY and FFA mass). Under these conditions, a scaled up esterification with an EE of 74.8% was conducted to obtain the crude diacylglycerol as a feedstuff for MD in subsequent trials.

Three immobilized lipases, Novozym 435, Lipozyme RM IM and Lipozyme TL IM, were screened for production of diacylglycerols by esterification of GLY with FFA from the r deodorizer distillate from vegetable oils [23]. Novozym 435 was selected from among the lipases due to the best performance in the esterification. In this lipase process, the preferred reaction conditions for lipase catalysis were: enzyme load 1.8 wt%, reaction temperature 60 °C, mole ratio 2.5 of FFA to GLY, reaction time about 6 h and pressure 1.5 or 0.5 kPa. Under these conditions, the content of DAG in crude diacylglycerols was reported to be about 50 wt% which was slightly higher than that of this

study. The optimal enzyme load for Novozym 435 was 1.8 wt% but for Lecitase Ultra in this study was 0.8 wt%, since Lipozyme RM IM lipase was immobilized whereas the Lecitase Ultra was in a free state. The immobilized lipase seemed to be recovered easily from the reaction mixture, but liquid Lecitase Ultra also had a potential to be recovered in a form of lower polar layer by centrifugation. The prices of immobilized lipases from Novozymes A/S, such as Lipozyme RM IM and Novozym 435, are 10–15 times that of Lecitase Ultra in China. Although the cost of production of DEO by immobilized lipases could be reduced by careful recovery of the enzyme, this new simple process with a low-price catalyst (Lecitase Ultra) seemed to be more commercially attractive. In addition, immobilization of the cheap Lecitase Ultra in a supported material should be attractive to reduce the cost when the process is commercialized.

Reaction Process of the Esterification Under the Selected Conditions

The esterification process was carried out under the optimal conditions except that the reaction time was prolonged to 36 h and is shown in Fig. 2. In the initial stage (the first 6 h) of the reaction, the contents of acylglycerols increased rapidly, and the DAG content of 26.7 wt% was achieved with a 1-h reaction time. However, the content of TAG increased slightly when compared with MAG and DAG, which agreed with a previous study reporting TAG formation occurring after the production of MAG and DAG [24].

In the second stage of the reaction (from 6–20 h), the contents of acylglycerols increased slightly and the curves became flat. The content of DAG reached its maximum of 42.2 wt% at 20 h. Although both DAG and MAG were all intermediate products during the esterification of FFA with GLY, DAG was relatively difficult to react with the excess GLY to produce TAG due to the higher molecular steric hindrance than MAG. Hence, the content of DAG reached a higher level than MAG. The contents of acylglycerols and FFA at around 20 h were relatively constant which seemed that the reaction had approached equilibrium.

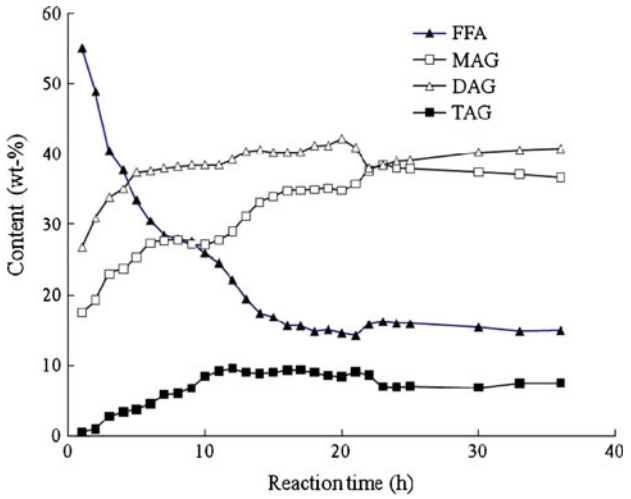

Fig. 2 The esterification reaction process of free fatty acid (FFA) with glycerol (GLY) using Lecitase Ultra. Reaction conditions: reaction time of 36 h, water content of 8 wt% (FFA and GLY mass), reaction temperature of 40 °C, and mole ratio of GLY to FFA of 2:1

However, when the reaction time was prolonged (from 20–22 h), the contents of DAG and TAG were slightly lower and the contents of MAG and FFA slightly lower simultaneously. DAG and TAG were hydrolyzed into MAG and FFA at this stage of the reaction (from 20–22 h). It was reported that the shear effect caused by high agitation speed decreases enzyme (lipase) activity [25], so we postulated that Lecitase Ultra was denatured to some extent by mechanical agitation for a long time, which changed the hydrophilic ability of the Lecitase Ultra and its absorption property of substrates so as to shift the reaction. In addition, although the added and the newly formed water by esterification could be removed by a reduced pressure, but the efficiency was dependent on the reaction temperature as well as the pressure. The presence of water in the reaction medium had a tendency to hydrolyze TAG and DAG by partially deactivated Lecitase Ultra.

In the final stage of reaction (from 22–36 h), the content of DAG was slightly increased while the contents of MAG and FFA were slightly decreased, which indicated that esterification of MAG with FFA to form DAG still occurred during this time period.

The unreacted FFA remained at 15.0 wt% at the end and the MAG content increased with reaction time continuously. Hence, the selection of reaction time should be a balance of a high content of DAG, a low content of byproducts and lower energy consumption.

Analysis for Acylglycerols by High Temperature GC

Figure 3a presents a gas chromatogram of a crude diacylglycerol obtained under reaction conditions which were reaction temperature 40 °C, 8 wt% water content (GLY and FFA mass), 6 h reaction time, 2.0 mol ratio of GLY and an enzyme load of 80 U/g (GLY and FFA mass), using a DB-1ht GC capillary column. The retention times and the contents of FFA and acylglycerols were determined by the standards calibration curves. The peaks of FFA (retention time 2.1–2.9 min), MAG (retention time 3.1–3.8 min), DAG (retention time 6.1–7.3 min) and TAG (retention time 9.4–10.2 min) were clearly separated, and the whole analysis time was accomplished within 11 min. Fagan et al. [26] reported on the development of a GC method with DB-1ht GC column for determination of individual

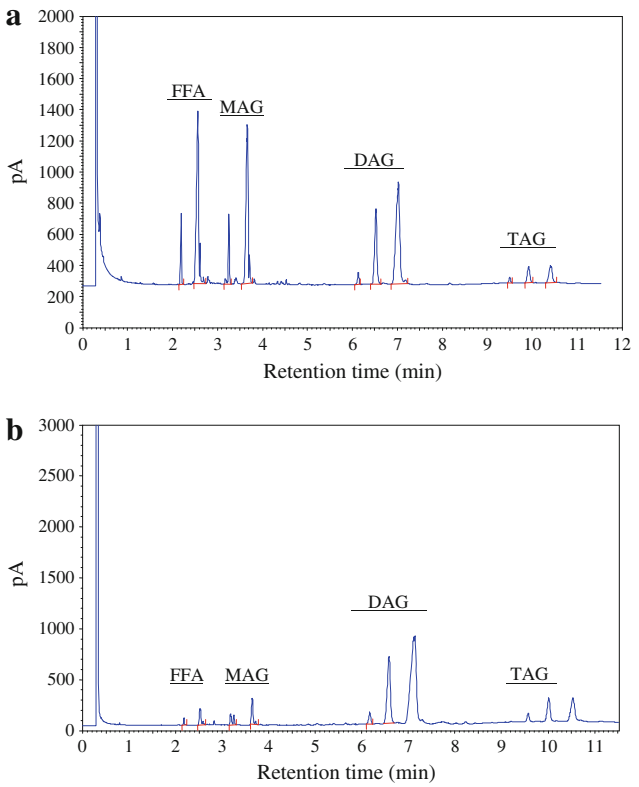

Fig. 3 Gas chromatogram of acylglycerols and FFA using DB-1ht capillary column (15 m × 0.25 mm × 0.10 μm). **a** Crude diacylglycerol, reaction conditions: reaction temperature 40 °C, water content 8 wt% (GLY and FFA mass), reaction time 6 h, mol ratio 2.0 of GLY and an enzyme load of 80 U/g (GLY and FFA mass). **b** Diacylglycerol-enriched oil (DEO) by molecular distillation at 170 °C. *FFA* Free fatty acid, *MAG* monoacylglycerol, *DAG* diacylglycerol, and *TAG* triacylglycerol

components of MAG and DAG in milk lipids with a whole time of 44 min. Lau et al. [27] investigated the reduction of column temperature by lowering the boiling points of acylglycerols using silylating reagent Bis(trimethylsilyl) trifluoroacetamide (BSTFA). Pretreatment of acylglycerols with BSTFA for GC analysis was also studies in our preliminary study (data not shown), which was proved to be not an essential step when using a high temperature gas chromatographic column in a short analysis time.

Preparation of DEO by MD

The results of the yield of DEO, removal efficiency, acid value and DAG purity in the distillates and residues at different temperatures by MD are described in Table 1. The yield of DEO decreased with increases in evaporator temperatures since much FFA together with MAG in the crude diacylglycerols was molecularly distilled into distillates at high temperatures. Removal efficiency increased with the increase in temperatures. The purity of DEO increased with temperature and reached a maximum of 83.1 wt% at 170 °C, and then slightly decreased to around 70 wt% at higher temperatures, since much DAG was entrained into the distillates at a high temperatures which decreased the purity and the yield of DEO. The removal efficiency of FFA reached over 96% at the evaporator temperature higher than 160 °C. For a compromise the yield and the DAG purity of DEO, the evaporator temperature of 170 °C was selected for removing FFA and MAG from crude diacylglycerols to production of the DEO. At this temperature, a DEO with a yield of 42.7 wt% and purity of 83.1 wt% was obtained, whose gas chromatogram was displayed in Fig. 3b. The ratios of *sn*-1,3-DAG versus *sn*-1,2 (2,3)-DAG of the DEO at 170 °C was 1.01. The selectivity of the lipase, the lipase carrier material, and the temperature of the reaction and purification contributed to the DAG ratio of isomers in the final product [28].

It was reported that FFA was removed as the distillate of MD from the soybean oil deodorizer distillate at an evaporator temperature of 125 °C [29]. The acid value of FFA composition of soybean oil measured was 202.7 mg KOH/g. However, the acid value of the distillate was only about 140 mg KOH/g at the evaporator temperature of 120–130 °C, because of a high amount of MAG presenting distillate. When the evaporator temperatures were in the range of 120–160 °C, the acid value of the distillates decreased rapidly, but remained relatively constant at over 160 °C, since MAG together with FFA was effectively removed at this temperature.

FFA Composition Analysis

The FFA compositions of the FFA from partial hydrolysis (starting material) and the DEO at 170 °C are shown in Table 2. Comparing with the starting material, the contents of saturated FFA (palmitic acid) and unsaturated FA (oleic acid, linoleic acid and linolenic acid) in the DEO remained almost unchanged, which indicated that the Lecitase Ultra showed non-selectivity towards the fatty acids between C16 and C18 during the esterification of FFA with GLY.

Conclusions

A new process of production of DEO by esterification of FFA with GLY using Lecitase Ultra was developed. The optimal conditions of esterification were found to be as follows: reaction temperature 40 °C, water content 8 wt% (FFA and GLY mass), reaction time 6 h, mol ratio of GLY to FFA 2.0, and an enzyme load of 80 U/g (FFA and GLY mass). Under these conditions, the EE of FFA was 74.8%. A high temperature GC was introduced to determine the

Table 1 The yield of DEO, acid value and DAG purity in the distillate and the residual at different evaporator temperatures by MD

Evaporator temperature (°C)	Yield of DEO (wt%)	Acid value (mg KOH/g)		Removal efficiency (%)	DAG purity (wt%)		MAG content (%)		TAG content (%)	
		Distillate	Residual		Distillate	Residual	Distillate	Residual	Distillate	Residual
The crude DAG	–	–	–	–	37.62		27.32		4.54	
100	83.6	169.5	40.28	34.1	2.76	43.9	6.30	31.02	0.12	4.73
110	71.8	157.2	18.17	74.5	3.53	49.9	9.70	33.33	0.39	4.89
120	60.1	149.2	12.68	85.1	3.86	57.5	15.4	30.25	0.32	6.35
130	55.0	134.5	5.46	94.1	4.70	65.3	22.9	24.79	0.37	7.06
140	48.9	117.1	4.82	95.4	4.72	72.2	31.4	14.81	0.35	8.23
150	46.0	102.1	3.29	97.0	4.79	75.4	35.1	10.82	0.43	7.73
160	45.5	104.1	3.61	96.8	4.95	77.3	34.3	11.01	0.31	8.10
170	42.7	100.9	3.05	97.5	5.59	83.1	35.0	7.35	0.45	10.2
180	43.5	104.1	3.59	96.9	6.62	79.5	34.1	9.59	0.56	8.05
190	43.0	101.1	3.87	96.7	7.57	77.3	34.2	9.84	0.57	9.68
200	37.5	95.90	4.36	96.8	12.26	73.3	34.1	11.48	0.64	10.5
210	33.5	85.30	3.34	97.7	13.73	71.6	37.0	7.81	0.62	11.4

Values are means of duplicates

DEO Diacylglycerol-enriched oil, *MD* molecular distillation, *MAG* monoacylglycerol, and *DAG* diacylglycerol

Table 2 The FFA composition of FFA from partial hydrolysis and the DEO by molecular distillation at 170 °C

Composition (wt%)	Palmitic acid	Oleic acid	Linoleic acid	Linolenic acid
FFA from partial hydrolysis	14.6	23.3	57.1	4.98
DEO	13.9	24.2	56.4	5.54

Values are means of duplicates

FFA Free fatty acid, *DEO* diacylglycerol-enriched oil

compositions of the FFA and acylglycerols within 11 min. MD was employed to produce DEO from the crude diacylglycerols. A DEO with a purity of 83.1 wt% and a yield of 42.7 wt% was obtained at 170 °C the evaporator temperature by one-step MD.

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