Preparation of High-Purity Trilinolein and Triolein by Enzymatic Esterification Reaction Combined with Column Chromatography

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Abstract: High-purity trilinolein and triolein were prepared by Novozym 435-catalyzed esterification reaction combined with column chromatography purification in this study. Firstly, linoleic acid and oleic acid were respectively extracted from safflower seed oil and camellia seed oil by urea adduct method. Secondly, trilinolein and triolein were synthesized through Novozym 435 catalyzed esterification of glycerol and fatty acids. The best synthesis conditions were obtained as follows: reaction temperature 100°C, residual pressure 0.9 kPa, enzyme dosage 6%, molar ratio of glycerol to linoleic acid 1:3 and reaction time 8 h. Crude trilinolein and triolein were further purified by silica gel column chromatography. Finally, high-purity trilinolein (95.43±0.97%) and triolein (93.07±1.05%) were obtained.

Key words: trilinolein, triolein, enzymatic, esterification, high-purity

1 Introduction

Trilinolein and triolein are important chemical and pharmaceutical raw materials\(^1\). Trilinolein can be used as lubricants in the textile industry, and smoothing agent in metal processing industry. Triolein can be used as emulsifier, emulsifying stabilizer and wetting agents in food and cosmetics\(^2\)\(^-\)\(^4\). Trilinolein and triolein can be obtained by extracting them from natural products or by artificial synthesis method. However, the cost of extracting trilinolein and triolein from natural oils is too expensive for industry use.

At present, there are two kinds of triglyceride (TAG) synthesis methods such as esterification reaction and transesterification reaction. It is difficult for the transesterification reaction method to obtain high purity TAG\(^5\)\(^-\)\(^6\). A huge number of papers on TAG synthesis by esterification reaction method are published every year. Compared with traditional chemical esterification reactions, lipase-catalyzed esterification reactions have the advantages of elevated reaction rate, higher efficiency, higher purity of the product and environment friendly\(^7\). Lipase-catalyzed esterification reactions are especially suitable for food and medicine synthesis\(^7\). The esterification degree of enzyme catalyzed fatty acid and glycerol can reach above 95%. However, the purity of the TAG synthesized by esterification method was usually less than 90%, because of the presence of partial glycerides, such as monoglyceride (MAG) and diglyceride (DAG)\(^8\)\(^-\)\(^10\). Chemical structures of TAG, MAG and DAG were shown in Fig. 1. Liu studied the optimal synthesis conditions for enzymatic esterification synthesis of triglyceride, and under the optimal synthesis conditions the total content of triglyceride reached up to 90.77±0.85%\(^11\). However, because of low-purity oleic acid used as raw material, the obtained triglyceride was a mixture of several different kinds of triglyceride. Therefore, it is necessary to use high purity fatty acid as raw material to obtain high purity TAG.

Also, the crude TAG product could be further purified by removing the fatty acids (FFA), MAG and DAG. Commonly used purification methods are column chromatography and molecular distillation, which can purify the TAG by decolorization, deacidification and further enrichment of TAG\(^12\). However, high temperature molecular distillation was required for further purification of TAG, which would lead to oxidation and isomerization of TAG\(^13\). It is necessary to explore useful purification methods.

The objective of this paper was to research the synthesis and purification method of trilinolein and triolein. Firstly,

\textbf{Abbreviations}: TAG, triglyceride; MAG, monoglyceride; DAG, diglyceride; FFA, fatty acid
high purity linoleic acid and oleic acid were prepared by urea adduction fractionation. And then trilinolein and triolein were synthesized through Novozym 435 catalyzed esterification of glycerol and FFA. Finally, high-purity trilinolein and triolein were obtained after purification process.

2 Materials and methods

2.1 Materials

Safflower oil and camellia seed oil was purchased from China National Cereals, Oils & Foodstuffs Corporation (Beijing, China). Ethanol (95%), petroleum ether (boiling range: 60-90 °C), sodium hydroxide, anhydrous sodium sulfate, sodium chloride, hydrochloric acid, urea, ethyl ether, n-hexane, methanol, boron trifluoride and propane-diol were of analytical grade from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). n-Hexane was of chromatographically pure. Lipase Novozym 435 are purchased from Novozymes Corporation (Bagsvaerd, Denmark). Standards of linoleic acid, oleic acid, trilinolein, triolein, mono-, di- & triglyceride mix, and 37 fatty acid methyl esters for analysis were obtained from Sigma-Aldrich.

2.2 Preparation of linoleic acid and oleic acid by urea adduction fractionation method

Fatty acids mixture was prepared from safflower oil according to previous method[10]. Fatty acids mixture, urea and 95% ethanol were mixed into a 500 mL glass container as a molar ratio of 1:2:10, and then urea adduction was performed at 60 °C for 90 mins. When the reaction was complete, the container was put into the refrigerating circulation pump, for the urea to crystallize for 12 h at −10 °C. At last, high purity linoleic acid was separated after rotary evaporation according to previous method[10].

For oleic acid preparation, fatty acids mixture were prepared from camellia seed oil and then a two stage urea adduction fractionation was performed. Fatty acids mixture, urea and 95% ethanol were mixed as a molar ratio of 3:4:10, and then urea adduction was performed at 60 °C for 120 mins. When the reaction was complete, the container was put into the refrigerating circulation pump, for the urea to crystallize for 14 h at 3 °C. The obtained first stage urea adduction product was mixed with urea and 95% ethanol as a molar ratio of 1:2.75:10 for the second stage urea adduction. Then, the next urea adduction was performed at 60 °C for 2 h, followed by the urea crystallizing for 12 h at 0 °C. Finally, high purity oleic acid was obtained after rotary evaporation[16, 17].

2.3 Fatty acid analysis

The fatty acid composition was determined by gas chromatography (GC) after derivatization to fatty acid methyl esters with 2N KOH in methanol, according to IUPAC method[18]. The analysis of fatty acid methyl esters was performed on an Agilent 7890B GC (Agilent, USA) equipped with a BPX-70 capillary column (30.0 m × 320 μm × 0.50 μm, SGE, Australia) and a flame ionization detector (FID, Agilent, USA). Nitrogen was used as the carrier at a flow rate of 1.0 mL/min. The column temperature was initially held at 180 °C for 5 min before being programmed to reached 230 °C at a rate of 3 °C/min and was maintained isothermally for 15 min. The temperatures for the injector and the FID detector were set at 230 °C and 300 °C, respectively. Injections were performed using a split ratio of 1:50. Peaks in GC were identified by comparison with the reference standards.

2.4 Enzymatic synthesis of trilinolein and triolein

In this study, trilinolein and triolein were synthesized by Novozym 435-catalyzed esterification of glycerol and linoleic acid/oleic acid under vacuum. Reaction was conducted in oil bathed glass vessel, with volume of 100 mL, containing some amount of glycerol and linoleic acid/oleic acid (a total amount of 40 g) and initiated by the addition of 0.8 g Novozym 435 (2% of the total weight of substrates) with stirring at 400 rpm, 0.9 kPa and 100 °C. Samples were withdrawn periodically to monitor the composition of the reaction mixture.

2.5 Determination of trilinolein and triolein

The esterification products were analyzed by the same GC using a DB-1ht capillary column (30 m × 0.25 mm × 0.1 μm, Agilent, USA). The injected volume was 1 μL and the carrier gas was nitrogen at a flow rate of 4.41 mL/min. The initial column temperature was 100 °C and a temperature gradient was applied: from 100 °C to 290 °C at 50 °C/min, from 290 °C to 320 °C at 40 °C/min and then held at 320 °C for 8 min, from 320 °C to 360 °C at 20 °C/min and finally held at 360 °C for 15 min. The temperatures for the injector and
the FID detector were respectively set at 350°C and 400°C. Injections were performed using a split ratio of 1:20. Peaks in HPLC were identified by comparison with the reference standards. Acquisition and processing of data was accomplished by Agilent OpenLAB CDS software (Agilent, USA).

TAG purity and esterification degree were defined as Eq. (1) and Eq. (2) in this study:

\[
\text{TAG purity} = \frac{\text{TAG} \times 100}{\text{TAG} + \text{DAG} + \text{MAG} + \text{FFA}} \quad (1)
\]

\[
\text{Esterification degree} = (1 - \text{FFA} \times 100) \quad (2)
\]

2.6 Purification of trilinolein and triolein
In order to obtain highly pure trilinolein and triolein, silica gel column chromatogram was used to further remove MAG, DAG and FFA from glyceride mixtures. Trilinolein or triolein was weighted, dissolved in a small amount of n-hexane, and poured into the silica gel column. The elution gradient: 100:2.7 vol/vol volume; ethyl ether/n-hexane, 3 times column volume; ethyl ether/n-hexane (2/98; vol/vol), 5 times column volume; ethyl ether: n-hexane = 5:95, 10 times column volume; 100% ethyl ether, 10 times column volume. The effluent was collected and detected by the ultraviolet detector at 254 nm.

2.7 Statistical analysis
All experiments were repeated for triplication. Significant differences among means were accomplished by using an ANOVA procedure (p < 0.05).20

3 Results and discussion
3.1 Preparation of linoleic acid and oleic acid
Urea adduction fractionation method is commonly used to extract fatty acids. Urea adducts precipitate both saturated and monounsaturated hydrocarbon chains as urea complexes, leaving solubilized polyunsaturated fatty acids in the non-urea adduction fraction. With a linoleic acid content of 73% ~ 85%, safflower oil is an ideal raw material for preparing linoleic acid. Firstly the mixed fatty acids were made by saponification of safflower oil, and then high purity linoleic acid was directly prepared by urea adduction method. Table shows the fatty acid composition of the safflower oil as raw material and the linoleic acid as product. With a purity of 99.18 ± 0.94%, the linoleic acid can be used as raw material for the subsequent preparation of trilinolein.

Camellia seed oil is selected to be raw material for preparing oleic acid, with an oleic acid content of 74% ~ 85%. A two stage urea adduction method was used to prepare oleic acid. Firstly the fatty acids mixture were made by saponification of camellia seed oil. And then most saturated fatty acids were removed from the fatty acids mixture by the first stage urea adduction, and oleic acid with a purity of 88.72 ± 0.73% was prepared, as Table 1 shown. During the second stage urea adduction, urea and oleic acid formed a more stable crystal clathrate, but polyunsaturated fatty acids were difficult to be combined with urea. Therefore, the oleic acid was further purified by removing most polyunsaturated fatty acids. As Table 1 shown, contents of both C18:2 and C18:3 in oleic acid were dramatically decreased after the second stage urea adduction. The oleic acid (97.27 ± 0.98%) can be used as raw material for the subsequent synthesis of triolein.

3.2 Preparation of trilinolein
3.2.1 Influence of reaction temperature
Trilinolein was synthesized through Novozym 435-catalyzed esterification of glycerol and linoleic acid under vacuum.

As shown in Fig. 2 and Fig. 3, both trilinoleic content and esterification degree gradually increased with the reac-

| Fatty acid composition | Raw materials | Products |
|------------------------|--------------|----------|
|                        | Safflower oil | Camellia seed oil | Linoleic acid | Oleic acid |
|                        | (%)          | (%)       | (%)          | First urea adduction (%) | Second urea adduction (%) |
| C16:0                  | 6.47 ± 0.21  | 7.97 ± 0.25| 0           | 0.54 ± 0.06              | 0.36 ± 0.03               |
| C18:0                  | 1.38 ± 0.10  | 2.41 ± 0.22| 0           | 0                       | 0                       |
| C18:1                  | 14.82 ± 0.43 | 79.20 ± 0.69| 0.72 ± 0.05| 88.72 ± 0.73             | 97.27 ± 0.98             |
| C18:2                  | 76.93 ± 0.78 | 9.38 ± 0.36| 99.18 ± 0.94| 8.78 ± 0.36              | 2.08 ± 0.15              |
| C18:3                  | 0.26 ± 0.02  | 1.04 ± 0.14| 0           | 0.97 ± 0.08              | 0.29 ± 0.01              |
| C20:1                  | 0.14 ± 0.01  | 0         | 0           | 0                       | 0                       |

Table 1 Fatty acid composition of raw materials (safflower oil and camellia seed oil) and products (linoleic acid and oleic acid).

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The higher the temperature, the shorter the time it takes for the esterification to reach equilibrium. At reaction temperature of 100°C, both trilinolein content and esterification degree reached the highest level when the esterification reaction approached equilibrium. Under the condition of reaction temperature 100°C and negative pressure, the water produced by the esterification reaction is rapidly boiling and volatilizing, promoting the esterification reaction equilibrium to the right. Further raising the reaction temperature to 110°C ~ 120°C, there was no significant difference in trilinolein content and esterification degree (p > 0.05). However, at reaction temperature of 130°C, both trilinolein content and esterification degree dramatically reduced because of decreased reaction rate. That was probably attributed to the inactivation of enzyme at high temperature.

3.2.2 Influence of reaction time

Fig. 4 shows the time course of the esterification reaction at reaction temperature of 100°C and 0.9 kPa. As the time proceeded, the trilinolein content and esterification degree increased rapidly during the first 8 h (p < 0.001) and then grewed slowly until reached a balance. Both MAG content and DAG content increased quickly during the first 2 h (p < 0.05) and then decreased to 7.98% and 12.04%, respectively. The triglyceride content increased very slowly after 8 h of reaction (p = 0.012). However, too long reaction time can easily lead to oxidation and isomerization of triglyceride, as well as discoloration of the oil. At a best reaction time of 8 h, the reaction mixture was composed of 69.36% trilinolein, 7.98% MAG, 12.04% DAG and 10.62% FFA.

3.2.3 Influence of the molar ratio of glycerol to linoleic acid

As Fig. 5 shown, the molar ratio of glycerol to linoleic acid has substantial influence on esterification products composition. Increase in the ratio from 1:2 to 1:3, caused trilinolein purity to increase significantly (p < 0.001). The maximum trilinolein purity was 69.36% with glycerol and linoleic acid at a molar ratio of 1:3. However, the trilinolein purity decreased significantly when this ratio increased from 1:3 to 1:3.5 (p < 0.05). At a molar of 1:2 and 1:2.5, because of excessive glycerol in the system, both MAG content and DAG content were relatively higher than those with other molar ratio, resulting in the trilinolein purity less than 60%. At a molar of 1:3.5, the reaction is incomplete and the residual linoleic acid content is up to 20%,
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Due to the linoleic acid excess. Moreover, excessive linoleic acid caused the difficulties of the subsequent product deacidification. Therefore, the molar of glycerol to linoleic acid was fixed at 1:3 for the succeeding experiments.

3.2.4 Influence of enzyme loading

As shown in Fig. 6 and Fig. 7, the enzyme loading has significant effect on both purity of trilinolein ($p<0.05$) and esterification degree ($p<0.05$). Trilinoleic purity rised with reaction time rapidly at first 4 h, and gradually balanced at around 8 h. The content of trilinolein was only 64.38% at a reaction time of 8 h with 2% enzyme loading. Under the condition of enzyme loading 4%, the content of trilinoleic reached up to 69.36%. Further increases in the enzyme loading from 4% to 6%, caused trilinolein purity to increase significantly from 69.36% to 70.26%. Therefore, an enzyme loading of 6% was considered to be suitable.

3.2.5 Best conditions and trilinolein yield

The best conditions were obtained as follows: reaction temperature 100°C, reaction pressure 0.9 kPa, enzyme loading 6%, molar ratio of glycerol to linoleic acid 1:3 and reaction time 8 h. Under the best conditions, crude trilinolein product was obtained with a purity of 70.26 ± 0.74% and a esterification degree of 91.23 ± 0.88%.

3.3 Preparation of triolein

At reaction temperature of 100°C, reaction pressure of 0.9 kPa, enzyme loading of 6%, glycerol/oleic acid molar ratio of 3:1 and reaction time of 8 h, triolein was produced with a content of 68.19 ± 0.62% and a esterification degree of 91.64 ± 1.03%.

3.4 Purification of trilinolein and triolein

In addition to 70.26% trilinolein, the esterification reaction mixture also contains 7.69% MAG, 11.87% DAG and 10.18% FFA. To achieve highly pure trilinolein, the silica gel column chromatography was employed. The different components in esterification could be separated by gradient elutions of different polar eluent. Fatty acids were separated by ethyl ether/n-hexane (2/98; vol/vol), TAGs were eluted by ethyl ether/n-hexane (5/95; vol/vol), while MAG and DAG were washed out by 100% ethyl ether eluent. The effluent was collected and dissolved by reduced pressure distillation. Finally, purified trilinolein (95.43 ± 0.97%) was obtained.

Also, the crude triolein was further purified, and the
final purity was 93.07 ± 1.05%.

4 Conclusions
Preparation of high purity trilinolein and triolein by Novozym 435-catalyzed esterification reaction combined with column chromatography purification was reported in this study. Firstly, linoleic acid and oleic acid were respectively extracted from safflower seed oil and camellia seed oil by urea adduct method. Then, trilinolein and triolein were synthesized using glycerol and linoleic acid/oleic acid as raw materials by a Novozym 435-catalyzed esterification method. Crude trilinolein and triolein were further purified by column chromatography. Finally, high-purity trilinolein (95.43 ± 0.97%) and triolein (93.07 ± 1.05%) were obtained. Overall, the proposed improved multi-step process proved to be a prospective approach for the synthesis of trilinolein and triolein.

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