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Enzymatic Glycerolysis of Chinese Vegetable Tallow Fraction by Lipase and Study of the Mechanism*

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Abstract Glycerolysis of Chinese vegetable tallow (CVT) fraction was investigated using a 1,3-specific lipase from *Rhizopus arrhizus* as catalyst. Based upon a binary gradient HPLC with an evaporative light-scattering detector (ELSD), the contents of free fatty acids (FFA), monoglycerides (MG), diglycerides(DG) and triglycerides (TG) with their positional isomers during the glycerolysis were determined. The effects of water content and the ratio of glycerol to oil on the product distribution of glycerolysis were studied. Under the optimum reactant conditions: 250 units lipase per gram oil at 37 ℃ with 1:2 molar ratio of oil to glycerol in a solvent-free system, after 24 h reaction, the product consisted of 7.2% TG, 25.6% MG, 56.1% DG and 4.9% FFA (all by mass). Furthermore, the mechanism of glycerolysis was discussed in detail.
Keywords glycerolysis, *Rhizopus arrhizus*, lipase, Chinese vegetable tallow

1 INTRODUCTION

In food and pharmaceutical industries, monoglycerides (MG) and diglycerides (DG) are the most important emulsifiers. Current processes for the production of MG and DG are based on the interesterification of triglycerides (TG) with glycerol (glycerolysis) in the presence of inorganic catalysts at high temperatures (200—250℃)[1].

The replacement of inorganic catalysts by lipases (E.C. 3.1.1.3.) is a potential attractive way to prevent the formation of side products, reduce pollution and energy consumption due to mild reaction conditions.

There are several different enzymatic methods reported for the synthesis of MG and DG so far, such as selective hydrolysis of TG, alcoholysis of oils and fats, glycerolysis of TG, and direct esterification of glycerol with variable acyl donors. As reaction systems, reverse micelles, micro emulsions, water-organic biphasic systems and organic solvent have been investigated[2−5]. Among these methods, glycerolysis of fats and oils in a solvent free system is of most interest because 1 mol TG and 2 mol glycerol can yield 3 mol MG, theoretically. Moreover, toxic and expensive solvents and/or surfactants are avoided. Initiated by the work of Yamane *et al.*[6], there were many reports about process of solvent-free glycerolysis[7−10] but few in the literature discussed the glycerolysis mechanism. The main reason for this is that glycerolysis is complicated involving a large number of possible base reactions, equilibria and many isomers.

Chinese vegetable tallow (CVT) is a special local vegetable oil of China, which is characterized by a high 1,3-palmitin-2-olein content (about 70%). At present, it is cheap and used as raw material for soap, oil paint and other chemicals. It will be of interest to synthesize products with high commercial value such as mono- and diglycerides from CVT.

In this work, a lipase from *Rhizopus arrhizus* produced by our laboratory is tested as biocatalyst for the glycerolysis of refined Chinese vegetable tallow in a solvent free system aimed at the production of MG and DG. Moreover, the mechanism of glycerolysis in a solvent free system is discussed in detail.

2 MATERIALS AND METHODS

2.1 Materials

Rhizopus arrhizus strain was stored in our laboratory. Chinese vegetable tallow (CVT) was purchased from Nanjing Grain and Oil Company (Nanjing, China). Glycerol and other chemicals without special mention were of analytical grade.

Acetonitrile and Methylene chloride of HPLC grade were obtained from Caledon Laboratories Ltd. (Georgetown Ont., Canada). Acetic acid was of analytical grade and was obtained from Beijing Chemicals Factory (Beijing, China).

The following reference standards were purchased from Sigma Chemical (St. Louis, MO, USA): palmitic acid, oleic acid, 1-monopalmitoyl-rac-glycerol, 2-monopalmitoylglycerol, 1-monoolein, 2-monoolein, 1,2-diolein, 1,3-diolein, 1,2-dipalmitin, 1,3-dipalmitin, 1,3-dipalmitoyl-2-oleoyl-glycerol, triolein and 1,2-dioleyl-3-palmitoyl-glycerol.

The reference standards of 1-palmitin-2-olein and

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1-palmitin-3-olein were prepared by our laboratory and had a purity of >98% analyzed by GC.

2.2 Preparation of *Rhizopus arrhizus* lipase

The ingredients of culture medium for *Rhizopus arrhizus* were (by mass): soybean flour 4.0%, earthnut oil 1.0%, $MgSO_4$ 0.1%, K_2HPO_4 0.5% and $(NH_4)_2SO_4$ 0.2%. The culture was carried out at 26.5℃ in a shaker at 130 r·min^{-1} for 70 h[11]. The broth was centrifuged to remove the cells and the lipase in the supernatant was precipitated by three volumes of cold acetone (−10℃). The precipitation was washed three times by double volumes of cold acetone and subsequently dried at room temperature. The activity of lipase powder was measured to be 6000 U·g^{-1}.

2.3 Refinement of CVT

The crude CVT contained 70% 1,3-palmitin-2-olein (POP) and other high melting compounds such as tripalmitin. It could not be directly used as glycerolysis substrate in solvent-free system due to its high melting point (60—65℃). In this study, we isolated 1,3-palmitin-2-olein (POP) from crude CVT and used it as substrate for the glycerolysis.

First, the crude CVT was refined by crystallization in acetone: 100g crude CVT was dissolved in 600 ml acetone at 34℃, then the mixture was cooled to 21℃ at a rate of 2℃·h^{-1} and held at 21℃ for 10 h to ensure that the fractions with high melting point were thoroughly precipitated. The precipitate was removed by a vacuum filtration. The acetone in the filtrate was removed by using a rotary evaporator to obtain a colorless oil mixture. The colorless oil mixture was then collected and was re-crystallized in 1:1 (by volume) ether-hexane. The mixture was dissolved in 7 volumes of 1:1 (by volume) ether-hexane at 14℃, then cooled to 8 ℃ at a rate of 2℃·h^{-1} and held for 8 h, followed by removal of the formed precipitate using a vacuum filtration. The solvent was removed by a rotary evaporator, and the residue was refined CVT containing 96% POP.

The refined oil was treated with alumina to remove traces of MG, DG, FFA, oxidation products and water[12]. The melting point of the refined CVT was about 35℃.

2.4 Glycerolysis reaction

Refined CVT (10 g, 12 mmol) and an appropriate amount of glycerol (if not specially mentioned, mass fraction of 10% water) as well as free *Rhizopus arrhizus* lipase powder (250 U·g^{-1} oil) were added into a 50 ml conical flask with a rubber stopper. The mixture was incubated in a water bath with magnetic stirring (800 r·min^{-1}) at 37℃.

2.5 Hydrolysis reaction

The hydrolysis experiments were similar to that for glycerolysis, except that no glycerol was added: 0.23 g water and 10 g refined CVT (12 mmol) as well as free *Rhizopus arrhizus* lipase powder (250 U·g^{-1} oil) were added into a 50 ml conical flask with a rubber stopper. The mixture was incubated in a water bath with a magnetic stirring (800 r·min^{-1}) at 37℃.

2.6 Assay of enzyme activity

The lipase activity was determined by an olive oil emulsion method[13]. One unit of activity is defined as the amount of enzyme which liberates 1 micromole free fatty acid from olive oil per minute at 37℃.

2.7 HPLC/ELSD analysis

Quantitative analysis of the products was conducted by using an HPLC system from Alltech (USA) with an evaporative light-scattering detector (ELSD). The analytical column was Aalltima C_{18} (5 μm) (250 mm×4.6 mm I.D.).

The chromatographic conditions were as follows: flow-rate, 1.0 ml·min^{-1}; column temperature, 25℃; eluents of the mobile phase, acetonitrile-acetic acid (100:0.05, by volume) and methylene chloride (the eluent gradients were reported in Table 1); detector temperature, 70℃; Gain, 1; and pressure of nebulizing gas, 2×10^5 Pa. Ethyl acetate-chloroform (9:1, by volume) was used as a sample-dissolving solvent, and the injection volume was 20 μl. The identification of each of the glycerides and fatty acids was performed by comparing its retention time with that of the corresponding standard. Quantification of the glycerides and fatty acids was performed by the external standard method. A typical HPLC chromatogram of glycerolysis products is represented in Fig. 1.

Table 1 Binary gradient elution conditions

Time, min	Volume fraction of acetonitrile-acetic acid %	Volume fraction of methylene chloride %
0	100	0
4	100	0
12	70	30
18	70	30
30	30	70
40	30	70
44	100	0

3 RESULTS AND DISCUSSION

3.1 Effect of water content on the glycerolysis

To investigate the effect of water content on the glycerolysis reaction, water was added into glycerol to give different water content (by mass): 4%, 8%, 10%, and 12%. When the water content was lower than 8%, the glycerolysis could not be carried out adequately, *i.e.* this lipase-catalyzed reaction needed higher water content (Table 2). However, in previous reports the perfect water content was 3%—4%[7,10]. The difference was possibly related to the source and activity of the lipase used. The *Rhizopus arrhizus* lipase used in

this study was crude with a low activity, so it needed a larger amount of lipase powder to obtain the desired lipase activity, resulting in that a higher water content in the glycerolysis was required. The MG content reached the maximum at a water content of 10% (by mass). In the case of the DG content, an increase with increasing water content was observed. The total MG and DG reached the highest value when the water content was 10%.

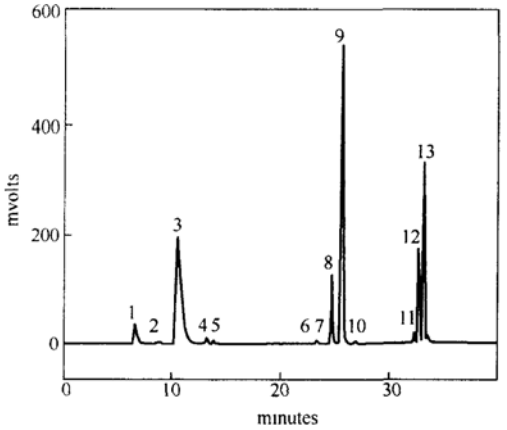

Figure 1 A typical HPLC chromatogram of glycerolysis products

1—2-monoolein; 2—1-monoolein; 3—1-monopalmitin; 4—oleic acid; 5—palmitic acid; 6—1,3-diolein; 7—1-palmitin-3-olein; 8—1-palmitin-2-olein; 9—1,3-dipalmitin; 10—triolein; 11—1,2-olein-3-palmitin; 12—1,3-palmitin-2-olein

Table 3 shows the effect of water content on the product distribution of the glycerolysis. The MG fraction of the glycerolysis product contained 1-monopalmitin, 1-monoolein and 2-monoolein. The content of monopalmitin was much higher than that of monoolein. The DG fraction of the glycerolysis product consisted of 1-palmitin-3-olein, 1-palmitin-2-olein as well as a little amount of 1,3-dipalmitin and 1,3-diolein (data not shown). When the water mass fraction in the glycerol was 10% and 12%, respectively, 1-palmitin-3-olein was predominant. However, when the water content was 8% (by mass), the content of 1-palmitin-3olein was lower than that of 1-palmitin-2-olein.

In the case of the TG fraction, it was interesting that 1-palmitin-2,3-diolein (POO) and triolein (OOO) were formed, resulting in the enrichment of oleic acid component in TG compared to the original POP. The content of POP in TG decreased with increasing water content.

3.2 Effect of molar ratio of glycerol/oil on the glycerolysis

In this study, the amount of glycerol varied while POP was fixed at 10 g to give desired molar ratios of glycerol to POP (Table 4). As the molar ratio of glycerol/oil increased, the MG content increased up to a molar ratio of glycerol/oil at 2.5:1, and above this point a slight decrease was observed. The content of DG decreased as the molar ratio of glycerol to POP increased. The total content of MG and DG reached a maximum at the molar ratio of 2:1. Though the content of POP was the lowest at 3:1 (by mole) of glycerol/oil, the FFA content was higher. The reason for this may be that higher content of glycerol in the original reactant would cause a high water content. Moreover, there was more glycerol left in the glycerolysis product since the total content of FFA, MG, DG and TG was only 81.4% (by mass).

Table 2 Effect of water content on the glycerolysis product

Water content, %	w_{FFA}, %	w_{MG}, %	w_{DG}, %	w_{DG+MG}, %	w_{TG}, %
4	0	0.3	1.9	2.2	80.1
8	3.8	20.2	37.6	57.8	27.2
10	4.9	25.6	56.1	81.7	7.2
12	8.6	19.3	56.5	75.8	6.9

Conditions: 250 units lipase g^{-1} oil at 37℃ with 1:2 molar ratio of oil and glycerol for 24 h.

Table 3 Effect of water content on the product distribution of glycerolysis

Water content, %	w_{MG}, %			w_{DG}, %		w_{FFA}, %		w_{TG}, %		
	1-MP	1-MO	2-MO	1,3-PO	1,2-PO	P	O	OOO	POO	POP
4	0.3	—	—	0.9	1.0	—	—	—	—	82
8	18.5	1.4	0.3	10.5	25.8	2.0	1.0	0.02	1.3	27.2
10	22.8	2.0	0.8	50.1	1.4	4.4	0.5	1.8	3.4	2.0
12	16.4	2.2	0.7	51.7	0.4	7.2	0.4	2.5	3.2	1.2

Note: P—palmitic acid, O—oleic acid, 1-MP—1-monopalmitin, 1-MO—1-monoolein, 2-MO—2-monoolein, 1,2-PO—1-palmitin-2-olein, 1,3-PO—1-palmitin-3-olein, OOO—triolein, POO—1-palmitin-2,3-olein, POP—1,3-palmitin-2-olein. Reaction conditions were the same as those in Table 2.

Table 4 Effect of molar ratio of glycerol/POP on the glycerolysis product

Gly/POP, mol/mol	w_{FFA}, %	w_{MG}, %	w_{DG}, %	w_{DG+MG}, %	w_{TG}, %
1.6/1	3.9	19.5	57.5	77.0	7.4
2/1	4.9	25.6	56.1	81.7	7.2
2.5/1	4.3	26.4	51.6	78.0	6.8
3/1	6.4	23.3	45.3	68.6	6.6

Note: Conditions: water content in glycerol, 10% (by mass); 250 units lipase per gram oil at 37 ℃ for 24 h.

Table 5 Effect of molar ratio of glycerol/POP on the product distribution of glycerolysis

Gly/POP, mol/mol	w_{MG}, %			w_{DG}, %		w_{FFA}, %		w_{TG}, %		
	1-MP	1-MO	2-MO	1,3-PO	1,2-PO	P	O	OOO	POO	POP
1.6/1	17.3	1.2	1.0	49.2	5.7	2.4	1.5	0.6	2.7	4.1
2/1	22.8	2.0	0.8	50.1	3.4	4.4	0.5	1.8	3.4	2.0
2.5/1	23.3	2.6	0.7	46.1	2.4	3.6	0.72	1.7	3.2	1.9
3/1	20.1	2.9	0.3	41.8	0.6	5.4	1.0	2.2	3.7	0.7

Note: Abbreviations were the same as those in Table 3. Reaction conditions were shown in Table 4.

Table 5 shows the effect of molar ratio of glycerol/oil on the product distribution of the glycerolysis. In the MG fraction, 1-monopalmin, 1-monoolein and 2-monoolein were found. In all cases, the MG were predominantly 1-monopalmitin and the content of 1-monoolein was higher than that of 2-monoolein.

In the glycerolysis products, DG was the major fraction. It seemed that the *Rhizopus arrhizus* lipase favored DG production. Besides the predominant 1-palmitin-3-olein, there were a little amounts of 1-palmitin-2-olein as well as 1,3-dipalmitin and 1,3-diolein (data not shown). The content of DG changed little with varying molar ratios of glycerol/oil. 1-palmitin-2-olein was the lowest at the molar ratio 3:1 of glycerol/oil.

The content of TG in the mixture was low in all cases. With increasing molar ratio of glycerol/POP, the POP content decreased and the OOO content increased. However, the total content of TG remained nearly unchanged.

In conclusion, using *Rhizopus arrhizus* lipase to catalyze the glycerolysis of a low melting CVT fraction was an effective and practical synthetic route for MG and DG production. The following part discussed the mechanism of glycerolysis in a solvent free system.

3.3 The mechanism of lipase-catalyzed glycerolysis

Glycerolysis is not a direct interesterification, but rather a hydrolysis of the ester bond in the triglyceride, followed by esterification of FFA and glycerol[12]. In order to analyze the glycerolysis mechanism, it is necessary to study the hydrolytic character of *Rhizopus arrhizus* lipase.

Figure 2 shows the time course of the hydrolysis of POP. In the hydrolysis products, the FFA fraction contained mostly palmitic acid. And the MG fraction contained 5.4% 2-monoolein, 1.3% 1-palmitin and 1.0% 1-monolein, while the DG contained 31.3% 1-palmitin-2-olein and 2.3% 1-palmitin-3-olein. From these results, two conclusions can be drawn: *Rhizopus arrhizus* lipase is 1,3-specific; 1-palmitin-3-olein is unlikely isomerized from 1-palmitin-2-olein, or this isomerization is difficult since the 1-palmitin-3-olein content is very low in these cases (Fig. 3).

Figure 4 shows the time course of the glycerolysis of POP. The glycerolysis reaction using 10 g POP was carried out under the optimum conditions (37℃, molar ratio of POP/ glycerol is 1:2, water mass fraction in glycerol of 10% and 250 units lipase·g^{-1} POP). After 24 h, the products from the Chinese vegetable tallow (CVT) fraction consisted of 7.2% TG, 25.6% MG, 56.1% DG and 4.9% FFA (by mass). The MG fraction of the glycerolysis product was enriched in palmitic acid, whereas, the fatty acid components of the TG were enriched in oleic acid, and the DG was mainly 1-palmitin-3-olein.

As the POP content decreased rapidly [Fig. 4(d)], the content 1-palmitin-2-olein [Fig. 4(c)] and 1-monopalmitin [Fig. 4(b)] increased rapidly in the first 5 h. Simultaneously, the palmitic acid content increased very quickly and remained constant in the following time, whereas the oleic acid content reached a maximum at a critical time of 12 h reaction [Fig. 4(a)]. As described above, the *Rhizopus arrhizus* lipase displayed sn-1,3 regioselectivity, and no 2-monopalmitin was detected. An explanation of this phenomenon may be drawn from the following mechanism, *i.e.* in the first 5 hours, the hydrolysis of POP and the incorporation of the resulting palmitic acid residue into glycerol yielding 1-monoplamitin were predominant (Fig. 5).

After 5 h of reaction, the decrease of POP slowed down, at the same time, the 1-palmitin-2-olein content started to decrease and the 1-palmitin-3-olein content increased quickly. At 24 hour, there was mainly 1-palmitin-3-olein, and less 1-palmitin-2-olein. Was 1-palmitin-3-olein formed from isomerization of 1-palmitin-2-olein? As described above in the hydrol-

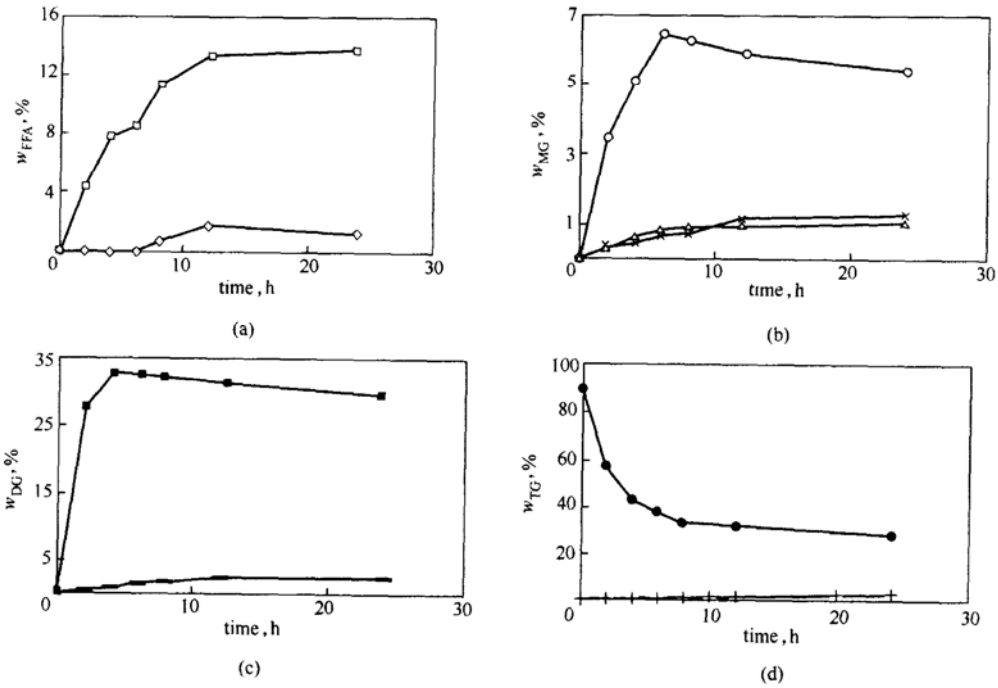

Figure 2 Time course of the products of hydrolysis
(a) FFA: ◇ oleic acid; □ palmitic acid; (b) MG: × 1-monoolein; ○ 2-monoolein; △ 1-monopalmitin; (c) DG: — 1-palmitin-3-olein; ■ 1-palmitin-2-olein; (d) TG: + 1,2-olein-3-palmitin ; ● 1,3-palmitin-2-olein

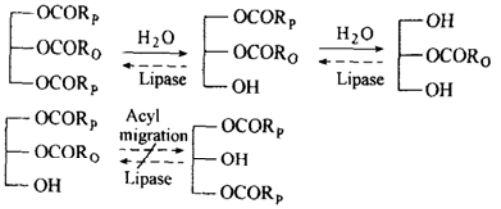

Figure 3 The mechanism of the hydrolysis of POP
[R_P: $CH_3(CH_2)_{14}$, R_O: $CH_3(CH_2)_7C_2H_2(CH_2)_7$]

ysis of POP, 1-palmitin-3-olein was unlikely to be isomerized from 1-palmitin-2-olein. Moreover, the glycerolysis reaction conditions were similar to that of the hydrolysis reaction except for the addition of glycerol. It may be concluded that 1-palmitin-3-olein is not formed from isomerization of 1-palmitin-2-olein, but from esterification of 1-monopalmitin and oleic acid or 1-monoolein and palmitic acid. Heisler et al. drew a similar conclusion in studying the mechanism of the lipase-catalyzed isomerization of 1,2-dipalmitin into the 1,3-isomer in glycerolysis[14]. The 1,3-diplamitin was not directly produced from isomerization of 1,2-diplamitin, but formed as follows: the first step is the hydrolysis of 1,2-dipalmitn followed by the isomerization of 2-monopalmitin into 1-monoester, which is then esterified into 1,3-dipalmitin.

In the case of diolein formation, it can only be formed from esterification. Due to steric hindrance, reaction 1 in Fig. 6 may be difficult to take place, and though trace of 1,2-diolein was determined during glycerolysis (data not shown), the formation of OOO during glycerolysis confirmed that reaction 1 in Fig. 6 must take place. It seems that the formation of OOO only can be explained by reaction 3 in Fig. 7.

According to these observations, it was shown that the esterifications of FFA and glycerides yielded by the hydrolysis of POP were predominant after 5 h. These reactions are illustrated with Figs. 6 and 7.

Figure 4(d) shows the compositional change of TG in the time course of the glycerolysis. It was interesting that POO and OOO were formed, which may be explained by Fig. 7.

Figure 8 represents the change of fatty acid composition of TG in the glycerolysis. In the first 4 h of reaction, TG contained POP only, the molar ratio of saturated fat component to unsaturated fat component (palmitic acid/oleic acid) in the TG was 2, but as the glycerolysis reaction proeeded, POO and OOO were formed. The molar ratio of saturated fat component to unsaturated fat component (palmitic acid/oleic acid) in TG decreased, and changed to 0.5 at 24 h, *i.e.* the TG was changed to predominantly unstaturated fat from a highly saturated oil. Thus, the TG fraction of the glycerolysis product may be a source of highly mono-unsaturated oil.

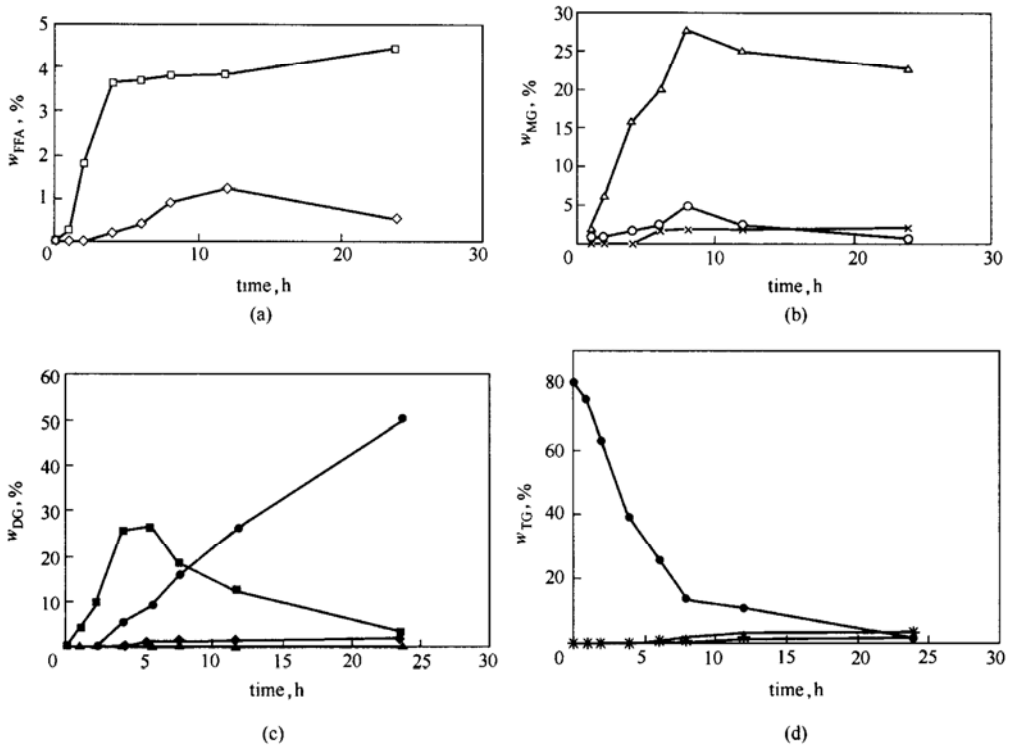

Figure 4 Time course of the glycerolysis of POP
(a) FFA: ◇ oleic acid; □ palmitic acid
(b) MG: × 1-monoolein; ○ 2-monoolein; △ 1-monopalmitin
(c) DG: ◆ 1,3-diolein; ▲ 1,3-dipalmitin; ● 1-palmitin-3-olein; ■ 1-palmitin-2-olein
(d) TG: * triolein; + 1,2-olein-3-palmitin; ● 1,3-palmitin-2-olein

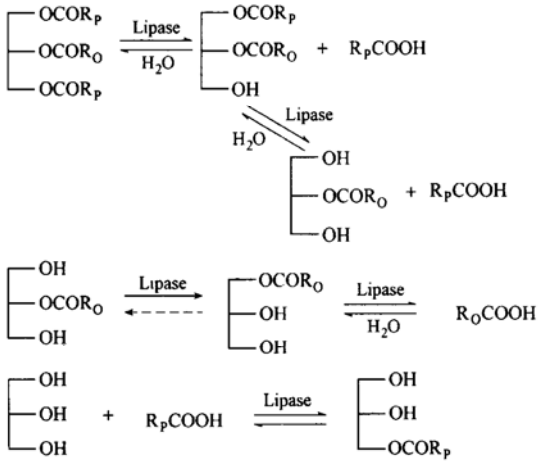

Figure 5 The main reactions in the first 5 h of the glycerolysis
[R_P: $CH_3(CH_2)_{14}$, R_O: $CH_3(CH_2)_7C_2H_2(CH_2)_7$]

4 CONCLUSIONS

Glycerolysis of a CVT fraction was investigated using a 1,3-specific lipase from *Rhizopus arrhizus* as catalyst. The lipase was proved to be effective and give a practical synthetic route of MG and DG production. The effects of water content and the ratio of glycerol to oil on the product distribution of glycerolysis were studied. Under the optimum reaction conditions: 250 units lipase g^{-1} oil at 37℃ with 1:2 molar ratio of oil to glycerol in a solvent-free system, after 24 h reaction, the product consisted of 7.2% TG, 25.6% MG, 56.1% DG and 4.9% FFA (by mass). The MG fraction of the glycerolysis product of CVT was enriched in palmitic acid, whereas the fatty acid

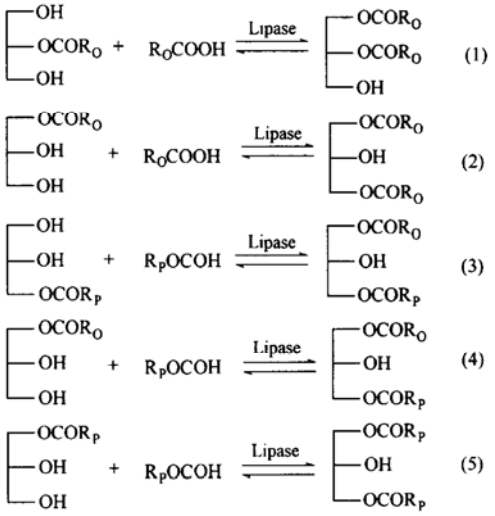

Figure 6 The main reactions after 5 h of glycerolysis reaction
[R_P: $CH_3(CH_2)_{14}$, R_O: $CH_3(CH_2)_7C_2H_2(CH_2)_7$]

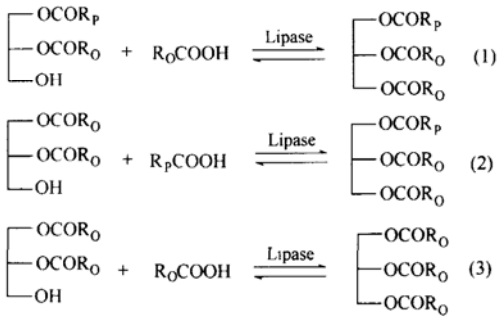

Figure 7 The Formation of POO and OOO
[R_P: $CH_3(CH_2)_{14}$, R_O: $CH_3(CH_2)_7C_2H_2(CH_2)_7$]

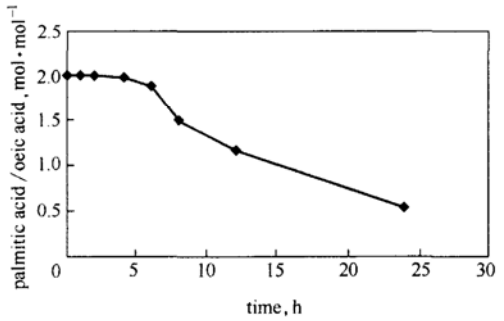

Figure 8 The Change of fatty acid components of TG during glycerolysis

composition of the TG fraction was enriched in oleic acid compared to the original oil, and the DG contained mainly 1-palmitin-3-olein. Furthermore, the mechanism of glycerolysis was discussed. Glycerolysis in solvent free system was not a direct interesterification, but rather the hydrolysis of the ester bond in the glyceride, followed by esterification of FFA and glycerol. Observations showed that in the first 5 hours, the hydrolysis of POP and the product palmitic acid with glycerol incorporated into 1-monoplamitin were predominant. 1-palmitin-3-olein was not formed from isomerization of 1-palmitin-2-olein, but from esterification of 1-monopalmitin and oleic acid or 1-monoolein and palmitic acid.

NOMENCLATURE

CVT	Chinese vegetable tallow
DG	diglyceride(s)
FFA	free fatty acid(s)
Gly	glycerol
MG	monoglyceride(s)
POP	1,3-palmitin-2-olein
TG	triglyceride(s)
w	mass fraction, %

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