One hour enzymatic synthesis of structure lipids enriched unsaturated fatty acids from silkworm pupae oil under microwave irradiation

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ABSTRACT: Silkworm pupa of *Bombyx mori* L. is considered as a valuable resource of unsaturated fatty acids (UFAs). Enzymatic transesterification that can produce the structured lipids (SLs) may be a desired approach to this resource, but excessive reaction time is unsatisfactory. An urgently need assist to reduce the reaction time. Microwave irradiation was employed to synthesize the novel SLs obtained under the molar ratio of silkworm pupa oil and oleic acid was 1/5 using solvent-free system catalyzed by Lipozyme RM IM (7%) at 60 °C in 1 h. The content of UFAs at *sn*-1,3 positon increased by 11.15% and the relative content of glycerol trioleate (OOO) in silkworm pupa oil (SPO) that obtained in microwave reactor was twice as high as that in the conventional reactors. Differential scanning calorimetry (DSC) and thermal gravimetric analyzer (TGA) were used to analysis the characteristics of the produced SLs. High performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) was employed to investigate the contribution to the fatty acids (FAs) after microwave irradiation. Variation on fatty acids distribution of enzymatic transesterification reaction caused by microwave irradiation to aid the addition of oleic acid has rarely reported.
KEYWORDS: transesterification, unsaturated fatty acids, Structure lipids, microwave irradiation, silkworm pupa oil, HPLC-ELSD

Introduction

Unsaturated fatty acids (UFAs) are a kind of fatty acids that make up body fat and essential fatty acids. They can regulate blood lipids, clear blood clots, immune regulation, maintain the retina to improve vision, and delay mental decline. So UFAs are important to human being health. There are a lot of resources containing UFAs can be collected, but among them just few of the UFAs can be assimilated by human body due to their distribution. Essential fatty acids of human are all UFAs and insufficient intake may lead to malnutrition and poor intellectual development of infants and young children [1]. However, it cannot be synthesized by the body and only be obtained from food. So the UFAs supplements have emerged. Artificially modifying natural oils and making it rich in UFAs has become a research hotspot in recent years. Chemical or enzymatic methods were reported to modify the natural oil [2]. The enzymatic synthesis has been widely studied due to its high efficiency, specificity, mild conditions, and low contamination. But in the conventional reaction system it takes a long time to change the fatty acid composition of the natural oil by exogenously adding oleic acid [3], which makes the natural oil easily oxidized and rancid in a long-time reaction process, resulting in a decrease in product quality. For example, under the optimal condition using a water bath involved a reaction temperature of 60 °C for 3h catalyzed by Lipozyme RM IM [3]. On the other research, at the optimum conditions, the OPO yield of 51.8% could be achieved in 4 h [4]. Several hours must be taken to obtain the suitable condition. Therefore, this experiment innovatively uses microwave radiation to solve the problem of long reaction time.

Microwave irradiation was known as enhancer that can increase the reaction rate. The microwave irradiation has been investigated that in the literature illustrating the breath of microwave chemistry, which cover such areas as heterocycles, natural products, peptides, catalytic reactions, and other medicinal compounds [5, 6]. Catalytic reaction can be enhanced on reaction efficiency due to the specific property of electromagnetic environment and heating principle by microwave reactor [7]. The instantaneous heating at molecular level is the specific property of microwave assisting organic synthesis while the conventional assistance cannot achieved. The microwave energy and molecules can be coupled directly in the reaction mixture which giving rise to the microwave irradiation generates efficient heating at the molecular level [8]. Reaction that require several hours under conventional conditions can be completed in few minutes. Under conventional heating (boiling water bath), transesterification step requires 40 min for triglycerides. However under low power (60 W) microwave irradiation, transesterification of triglycerides can be achieved in 60 s, respectively. [9].Microwave irradiation could be used to intensifies the extraction of lipids from yeast and meat [10]. There are few reports about the use of microwave enhanced esters

exchange reactions and there is rarely report on fatty acids distribution. Microwave irradiation was applied to assist the present study and it was expected to solve the problem of long reaction time.

Silkworm (*Bombyx mori.* L) pupa oil (SPO) was ignored for many years in China. In fact the previous research [11, 12] showed that SPO enriched with UFAs, and α-linolenic acid (ALA) [13]. However, polyunsaturated fatty acids (PUFAs) play an important role in human diet and physiology [14]. The mutritional value of SPO has attracted considerable attention for dietary and pharmaceutical [15]. On the other hand, the consumption of silkworm pupa as a source of human food is controversial because of sensitization. Another way of assimilating the silkworm pupa was been found. In the previous research, ultrasonic and water bath heated shaker and other auxiliary means were used to enzymatically catalyze the fatty acid composition of silkworm pupa oil. However, in the above studies, it was found that such enzymatic reactions take a long time and are urgently needed. A method that can shorten the reaction time, microwave irradiation may be an auxiliary means that we need.

In the present study, the FAs in the SPO were redistributed as far as possible through transesterification. Lipozyme RM IM which is a strict s*n*-1,3-specificity in many reactions with more regioselective [16] was used for enzymatic transesterification under the microwave processing conditions. By optimizing the substrate ratio, the enzyme content, microwave reaction temperature and microwave power, the experimental results were presented. Furthermore the effect of microwave irradiation on fatty acid distribution has also been studied.

Methods and Materials

Experiment material and instruments

The microwave irradiation systems is constituted with CEM Corporation (MARSX，CEM, USA). Gas chromatography (GC) on HP-INNOWAX , 30 m × 0.25 mm ids , 0.25-μm capillary column (Model 6820 N ; Agilent) installed on an Agilent Technologies gas chromatograph equipped with a Hewlett Packard 3396 Series II integrator and 7673 controller , a flame ionization detector , QA/QC nds certify system and split injection (Agilent Technologies Inc, Santa Clara, CA). Differential scanning calorimeter is DSC 8500 from PerkinElmer and Thermogravimetric analysis is Pyris 1 TGA from PerkinElmer. High performance liquid chromatography (HPLC) is Agilent 1260 with an evaporative light scattering detector (ELSD, Agilent 1260).

Desilked silkworm pupa were collected in May 2009 at Jiangsu Province of China and it was been authenticated as *Bombyx mori.* L pupae (Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China). The sample was dried at 60 °C in a DHG9240A drying oven (Shanghai Yiheng Scientific instruments, Shanghai, China), powered by a herb disintegrator (Qinzhou Sanyang Package Equipment, Qinzhou, China) and sieved (60 esh) [17]. Lipozyme RM IM (Novo Nordisk A/S, Denmark) was purchased from Novo Nordisk A/S (Bagsvaerd, Denmark). Porcine pancreatic lipase and the standard TAGs of 1, 2, 3-trioleoylglycerol (OOO), 1,2,3-tripalmitoylglycerol (PPP), 1,2,3-trilinoleoylglycerol (LLL) and 1,2,3-trilinolenoylglycerol (LnLnLn) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The conventional reagents were purchased from the national pharmaceutical group.

Acidolysis by lipase Lipozyme RM IM

Silkworm pupa oil (SPO) was extracted from desilked silkworm pupa. Lipozyme RM IM was mixed with reactants (including SPO and oleic oil) and they were put into 50 mL centrifuge tube. To obtain the maximum UFAs constant molar ratios, enzyme loadings, microwave temperatures, microwave power and the reaction time were ranged from 1/1 to 1/9, 3% to 9%, 40 °C to 60 °C, 30W to 200W and 30 min to 180 min respectively. The SLs products were collected and stored at 4 °C.

Hydrolysis with pancreatic lipase

TAGs were blended with Tris-HCl (1 M，pH=8.0), sodium cholate (0.05% v/v) and calcium chloride (2.2%) in 10 mL centrifuge tube and vortexed for 2 mins, the mixture was placed in a 40 °C water bath for 3 mins. Pancreatic lipase was added to the mixture and the mixtures were placed in a 40 °C water bath for 3 mins after vortex. HCl (6 M) and diethyl ether were added to the mixture. The new mixtures after shaking centrifuged at 10000 rpm for 3 min. The ether layer was collected after dehydration with anhydrous sodium sulfate, and evaporated to 1/3 of the volume using nitrogen gas.

Separating FA at the Sn-2 position

The ether layer mentioned above were separated on TLC (Silica gel GF254 TLC plate, 10×20 cm) with the n-hexane/ether/ formic acid (60:40:1.6, v/v/v) as the developing solvent. As shown in Figure 1, the Separated FAs at the *sn*-2 position was in the red block area at the plates after staining in a cylinder of iodine. The lowest band area was collected with a centrifuge tube for the methyl esterification after scraping.

Methyl esterification

Methyl esterification was performed to transform FAs into FA methyl esters (FAMEs) [18]. 2 mL NaOH-CH_3OH (0.5 mol/L) and 1.5 mL N-hexane were mixed with the SL products (150 μL) in a 10 mL centrifuge tube. The mixture was gently shaken and heating in a 60 °C water bath for 1 h and then centrifuged at 10000 rpm for 5 min. Thus, FAs were transformed into FA methyl esters [18].

Analysis of fatty acid composition using gas chromatography

The temperature of the injector and detector were set as 250 °C and 280 °C respectively, with a sample volume of 1 μL, a column head pressure of 200 kPa and a split ratio 50:1. GC results were tabulated and the content of fatty acids at *sn*-1,3 position were calculated using the following equations:

$$\mathrm{FA_{(i)}\ incorporation\ at}\ sn\text{ - }1,3\ \mathrm{position} = \frac{3 \times \sum \mathrm{FA} - \mathrm{FA_{(i)}\ at}\ sn\text{ - }2\ \mathrm{position}}{2} \times 100\% \quad (1)$$

HPLC-ELSD condition for TAG analysis

For the TAG analysis, 1 mL dilute standard TAG filtered with 0.45 μm membrane were injected into a 1.5 mL sample bottle. MSLs and CSLs which were products in microwave reactor and shaker bath reactor were diluted with acetonitrile by 200-fold. The chromatographic separation was carried out using the two stages of acetonitrile/dichloromethane, 85% of acetonitrile from 5 min to 30 min and 30% of acetonitrile from 30 min to 35 min [19]. The flow rate was 1.0 mL/min and the column temperature was 45 °C. The gas flow rate of ELSD was 1.6 slm, the evaporator and nebulizer temperature were set as 50 °C and 45 °C respectively.

Significance level analysis

All single factor experiments were investigated by using the R Project for ANOVA. By using the R Project to analyze the significant levels of each single factors when the significance level was less than 0.05, which indicates that the factors have a significant effect on the results.

Results and discussion

Effect of Transesterification condition on TAGs

Table 1 showed that the highest content of UFAs at *sn*-1,3 position was 83.11 ± 1.28% when the substrate ratio was 1/5 at 2.5h. Under the same condition the highest content OA (41.77 ± 0.56%) at *sn*-1,3 position was obtained and the PA content at *sn*-2 position was 14.61 ± 2.50%. The lower yields obtained with higher substrate ratio can be due to the binding extent between enzyme and the substrate since increasing the amount of OA actually prejudice the catalytic efficiency of enzymes. It was reported that 1/8 was the optimal condition for production of structured lipid 1,3-dioleoyl-2-palmitoylglycerol from tripalmitin and oleic acid [20]. Upon considering the content of UFAs and the downstream processing costs, the suitable amount of substrate should be selected as 1/5.

The highest content (reaching 73.82 ± 0.13%) of UFAs was obtained with 7% of enzyme loading at 2h in Table 2 while others lead to content between 65 and 70%. Furthermore, under the same condition the content of OA at *sn*-1,3 position and PA at *sn*-2 postion also reached the higher content (33.34 ± 3.75%, 19.54 ± 6.46%) than other enzyme loading. At the same hours, the content was increasing with the enzyme loading. The acyl migration could heighten with the increasing enzyme loading [21]. However the content obtained with 9% of enzyme loading was lower than 7%. The decline may be caused by the high concentration of free fatty acids or the ionized carboxylic acid groups which may acidify the lipase and cripple the activity of the enzyme [22]. 7% of enzyme loading was considered as the suitable condition.

Table 3 shows the highest content of UFAs at *sn*-1,3 and PA at *sn*-2 position were reaching 77.89 ± 2.20% and 28.29 ± 5.14% respectively under 50 W of microwave power at 1h. In addition the highest content of OA at *sn*-1,3 was obtained with 30 W of microwave power while the approximate content was acquisition under 50 W of microwave power at 1h. The lower content obtained with higher microwave power can be due to the microwave energy could cause the molecular roation [23] which prejudiced the activity of enzyme. 50 W of microwave power was considered as the suitable condition for enriching the UFAs. Table 4 showed that the highest content of OA (51.42 ± 1.96%) and UFAs (100.35 ± 6.45%) at *sn*-1, 3 position was obtained with 60 °C of microwave temperature. Mass transfer efficiency increases with the temperature increasing, while the excessive temperature can be inactivation of the enzyme. Therefor the suitable condition was considered as 60 °C for enriching the UFAs.

After the significance level analyzed, it suggested that the substrate ratio, enzyme loading, microwave power and temperature have the significant effect on the content of UFAs, OA and PA.

Table 1 Effect of substrate molar ratio on positional distribution of OA, PA and UFAs in MSLs

Time (h)	OA content at *sn*-1,3 (%)					PA content at *sn*-2 (%)					Unsaturated fatty acids content at *sn*-1,3 (%)				
	1/1	1/3	1/5	1/7	1/9	1/1	1/3	1/5	1/7	1/9	1/1	1/3	1/5	1/7	1/9
0.5	25.75±1.61	27.45±1.18	30.53±0.31	33.96±1.15	36.45±1.88	2.56±0.14	5.93±1.12	6.16±0.45	9.93±1.63	13.35±3.68	67.44±0.20	68.73±2.84	69.32±0.02	74.34±1.20	78.63±2.99
1	30.39±0.37	30.99±1.38	32.90±2.23	35.91±1.85	40.62±2.05	3.23±0.17	4.67±0.62	7.33±0.77	9.05±2.25	15.48±0.48	67.87±0.78	70.13±0.61	74.79±6.42	77.16±2.33	82.17±1.41
1.5	32.38±1.62	31.25±0.37	35.19±0.80	37.35±1.39	38.38±0.89	3.30±0.22	5.39±0.49	11.09±0.28	8.79±2.54	14.36±0.98	68.66±0.78	70.02±1.19	75.03±1.69	76.72±0.44	80.87±0.01
2	33.59±2.05	31.19±0.10	35.44±0.61	37.41±0.47	38.24±1.89	4.59±0.25	5.63±0.54	9.86±0.83	11.41±1.91	13.93±5.29	69.20±0.56	70.40±0.75	75.64±0.96	79.42±1.34	79.09±2.70
2.5	29.67±0.58	31.68±0.23	41.77±0.56	39.89±2.12	40.09±2.15	5.06±0.15	7.22±0.42	14.61±2.50	12.56±6.61	12.29±0.73	70.28±0.52	71.59±0.49	83.11±1.28	81.49±5.01	81.14±2.76
3	23.35±5.66	32.78±0.38	35.75±0.32	38.06±1.47	41.90±1.40	6.89±1.47	5.37±0.28	8.59±1.23	14.86±3.03	13.67±1.70	72.90±1.30	71.46±0.18	76.21±0.29	79.95±1.16	83.10±0.96

Table 2 Effect of enzyme loading (%) on positional distribution of OA, PA and UFAs in MSLs

Time (h)	OA content at *sn*-1,3 (%)				PA content at *sn*-2 (%)				Unsaturated fatty acids content at *sn*-1,3 (%)			
	3	5	7	9	3	5	7	9	3	5	7	9
0.5	30.87±0.74	28.53±2.43	25.49±1.84	28.38±2.79	14.35±2.95	17.81±1.94	16.00±2.96	17.10±1.13	67.70±0.43	70.96±2.52	72.42±3.25	71.37±0.41
1	30.16±1.08	28.27±0.18	33.15±2.55	31.58±1.60	11.83±2.30	11.57±0.62	25.70±1.98	14.85±0.15	67.28±0.94	68.13±0.44	74.67±2.50	71.49±0.15
1.5	30.44±0.27	31.40±1.38	31.05±0.55	31.35±1.09	10.60±0.87	14.53±1.28	18.83±4.88	15.55±4.04	67.52±0.58	70.02±2.94	72.52±2.34	70.55±2.37
2	33.21±0.80	31.14±0.20	33.34±3.75	33.70±0.45	16.51±3.19	11.15±0.78	19.54±6.46	12.42±1.57	69.99±1.52	69.91±0.45	73.82±0.13	69.78±0.99
2.5	33.02±1.71	30.56±0.30	33.24±0.81	31.93±1.36	13.14±3.33	11.61±1.37	13.58±2.90	11.22±0.72	68.42±0.37	70.79±0.63	69.02±3.24	71.57±0.46
3	33.12±0.28	30.55±1.10	31.29±0.26	32.98±5.48	13.32±1.00	11.16±0.25	12.75±3.04	11.83±3.63	70.27±0.36	70.53±0.30	71.04±1.75	73.33±1.05

Table 3 Effect of microwave power (W) on positional distribution of OA, PA and UFAs in MSLs

Time (h)	OA content at *sn*-1,3 (%)					PA content at *sn*-2 (%)					Unsaturated fatty acids content at *sn*-1,3 (%)				
	30	50	100	150	200	30	50	100	150	200	30	50	100	150	200
0.5	29.77±0.56	31.63±2.21	30.75±0.78	32.21±1.59	29.54±3.60	7.75±1.75	11.77±3.77	14.62±4.05	16.82±2.44	12.71±3.47	67.04±1.03	65.97±2.66	69.78±0.06	70.92±2.18	67.95±6.50
1	36.09±0.98	35.21±0.46	33.07±5.02	28.50±1.28	27.80±3.93	23.79±1.73	28.29±5.14	20.21±5.58	19.20±5.02	20.00±1.76	74.77±2.36	77.89±2.20	75.23±0.34	64.44±2.55	65.12±7.22
1.5	34.57±0.17	29.74±0.21	30.27±2.85	30.72±1.47	30.36±3.56	21.43±0.98	20.68±3.91	12.73±0.89	17.46±4.75	17.20±3.54	73.56±1.09	62.61±0.76	68.18±2.42	68.73±5.06	70.56±5.84
2	31.82±0.66	29.09±1.42	29.69±0.79	28.95±2.51	30.79±1.82	14.95±2.38	19.81±3.36	10.77±1.94	14.19±4.76	17.04±1.35	70.23±1.37	64.61±2.31	67.31±2.15	67.00±1.65	72.35±0.52
2.5	33.06±2.13	32.67±0.91	31.88±2.28	31.93±0.20	32.02±1.02	15.90±3.87	22.19±4.65	11.40±1.74	16.55±0.67	18.90±1.30	70.82±3.97	74.10±0.10	67.26±0.62	72.94±0.44	73.62±0.72
3	34.44±2.29	33.91±1.18	30.20±1.32	31.10±0.91	30.75±1.60	14.79±4.52	21.25±2.00	9.23±1.70	14.41±1.39	18.00±0.86	72.90±3.70	69.61±2.91	67.56±0.58	71.30±0.59	71.05±3.54

Table 4 Microwave temperature (℃) on positional distribution of OA, PA and UFAs in MSLs

Time (h)	OA content at *sn*-1,3 (%)					PA content at *sn*-2 (%)					Unsaturated fatty acids content at *sn*-1,3 (%)				
	40	45	50	55	60	40	45	50	55	60	40	45	50	55	60
0.5	37.23±2.81	42.39±5.50	31.06±5.81	35.63±2.71	38.17±4.88	40.29±5.73	50.38±8.13	60.16±2.73	47.16±7.07	47.28±9.43	79.10±6.68	88.71±9.96	67.33±1.14	84.20±1.24	83.93±9.69
1	38.51±1.59	37.02±4.68	37.28±5.23	34.79±6.06	40.23±4.59	45.89±3.88	49.83±7.31	59.71±3.45	42.19±2.26	47.33±6.88	82.30±4.43	87.67±2.61	81.27±1.85	80.13±1.63	85.46±8.51
1.5	38.38±3.83	46.03±1.12	41.41±3.45	32.80±1.06	43.93±3.32	52.48±2.87	50.33±4.69	56.49±8.05	31.51±3.21	56.53±2.24	81.01±5.53	89.99±7.63	86.91±4.15	80.37±1.36	95.01±5.94
2	31.51±1.50	34.37±0.89	37.96±4.03	40.92±3.28	45.16±0.21	40.41±1.30	52.73±1.5.2	56.03±1.5.74	31.30±0.12	57.30±5.97	73.24±2.73	80.89±0.63	84.23±6.64	84.28±5.80	95.50±0.83
2.5	33.92±1.31	37.76±2.85	43.68±1.88	39.44±4.03	45.53±1.60	28.52±1.93	42.11±5.29	55.90±8.42	30.12±7.92	55.74±7.91	75.91±4.64	81.16±7.67	93.13±3.89	80.76±0.04	96.11±2.39
3	36.89±1.51	47.58±3.06	42.14±4.99	34.95±2.08	51.42±1.96	39.64±5.90	48.70±1.28	49.84±1.4.44	28.85±7.84	52.28±2.99	82.52±0.56	93.64±7.00	88.12±7.71	77.29±3.03	100.35±6.45

Table 5 The lipid content in the mixture before and after the reaction and in the mixture under the conventional reaction.

Fatty acid	C16:0	C18:1	C18:2	C18:3
		Total fatty acid content (%)		
SPOSLs[a]	24.55±0.23	34.72±0.06	5.58±0.02	35.15±0.15
MSLs[b]	24.71±1.04	35.76±1.23	8.32±0.25	31.20±0.43
ESLs[c]	24.84±0.65	34.58±0.23	5.54±0.05	35.04±0.37
CSLs[d]	11.85	3.86	51.61	12.79
SSLs[e]	21.07±0.54	32.52±0.22	6.56±0.31	30.96±2.2
		Sn-2 fatty acid content (%)		
SPOSLs[a]	4.77±2.13	40.65±0.39	5.31±0.16	49.27±1.62
MSLs[b]	18.41±2.53	38.52±2.62	4.78±0.85	34.65±0.79
ESLs[c]	3.86±0.41	40.49±1.02	5.53±0.14	50.12±0.73
CSLs[d]	3.59	2.22	42.06	42.38
SSLs[e]	3.46±0.23	36.32±1.23	6.61±0.74	54.02±0.61
		Sn-1, 3 fatty acid content (%)		
SPOSLs[a]	34.43±1.39	31.75±0.27	5.72±0.10	28.09±1.03
MSLs[b]	28.35±3.13	33.47±0.01	10.10±0.43	29.30±1.20
ESLs[c]	35.33±1.05	31.62±0.78	5.54±0.09	27.50±0.60
CSLs[d]	16.43	3.68	48.09	28.03
SSLs[e]	30.49±0.74	28.72±2.21	6.63±0.75	22.93±1.35

[a] The extracted oil from silkworm pupa.
[b] Fatty acids composition of silkworm pupae oil transesterification product SLs under the optimum condition in microwave reactor.
[c] Adding the enzyme (RM IM) without oleic acid into the silkworm pupae oil under microwave irradiation.
[d] Fatty acids composition of silkworm pupae oil transesterification product SLs under the optimum condition from Zhao et al, 2015.
[e] The silkworm pupae oil used in shaker bath reactor.

Composition and distribution profits of SLs from silkworm pupa oil

Table 5 shows the predominant fatty acid of MSLs was OA (35.76 ± 1.23%) followed by ALA (31.20 ± 0.43%), PA (24.71 ± 1.04%) and LA (8.32 ± 0.25%). In *sn*-2 position of the MSLs, OA (38.52 ± 2.62%) was the major species, followed by ALA (34.65 ± 0.79%)，PA (18.41 ± 2.53%) and LA (4.78 ± 0.85%). After the microwave reaction, the aggrandize was been found that the content of PA at *sn*-2 position was increased from 4.77 ± 2.13% to 18.41 ± 2.53%, on the other hand the SLs produced though the heat bath reactor was not found this aggrandize at *sn*-2 position of CSLs. In human breast milk, over 60% of saturated fatty acids (SFAs) were detected at the *sn*-2 position and PA constitute the major [24]. and in the SFAs at *sn*-2 position of human breast milk, PA is the main ingredients which is a helpful composition for obesity and hyperlipidemia. For the human body, TAG with higher content of PA at *sn*-2 position and SFAs at *sn*-1,3 position is very nutritive [25].

The fatty acid composition of SPO following transesterification under optimum conditions in microwave irradiation was compared with the SLs in shaker bath reactor (Table 5). Lipozyme RM IM was known as a lipase with strict *sn*-1,3-specificity in many reaction, but to some extent, it can attach an influence on the PA content at *sn*-2 position that increasing by 2.7% in shaker water bath reactor [26]. By the way the microwave irradiation can enhance the reaction, not just the strict *sn*-1, 3 specificity, but also this influence. Furthermore, the microwave-heated favors the presence of saturated fatty acids [27]. So all of this could be the reason of the PA content aggrandize at *sn*-2 position, but to confirm the reason of this aggrandize, further experiments are needed to verify this phenomenon.

DSC characteristic of different SLs

Melting and crystallization characteristics of SPO and SLs in microwave reactor and shaker bath reactor were detected (Figure. 1 A, B). These properties are considered as good indicators of absorption of the body's ability to absorb [29]. When the fats with melting points lower than physiological temperature (36.6-37.3°C) can be quickly absorbed and emulsified. The melting point of MSLs, CSLs and SPOSLs were all lower than this temperature, at 2.46°C, 6.12 °C and -5.78°C, respectively (Figure. 1A). Crystallization characteristics of milk fats, although having little meaning for digestion, are of great importance for application purposes. The starting crystallization temperature of MSLs CSLs and SPOSLs were 2.14°C, 2.39 °C and 3.48°C, respectively (Fig. 1B) suggested that they could be stored at cold temperatures without inactivation. Above all this results indicated that the enzymatic reaction under microwave reactor had changed the melting and crystallization properties of SPO and got lower melting point and more suitable crystallization properties compared to enzymatic reaction under shaker bath reactor.

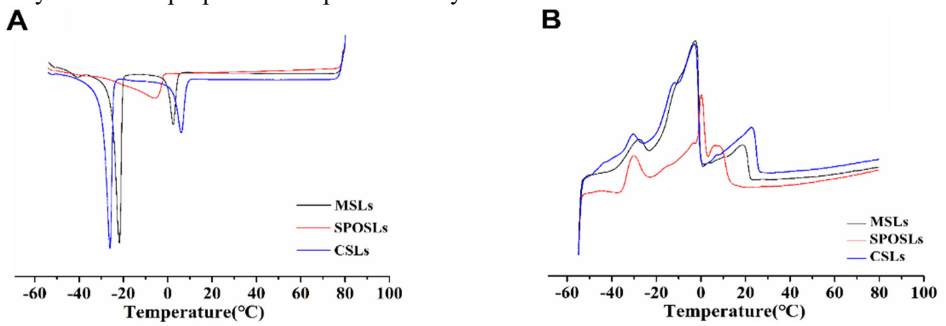

Fig. 1. Melting (A) and crystallization (B) characterization of SLs in different reactors. MSLs is the product under the optimum condition in microwave reactor. SPOSLs is the crude silkworm pupae oil. CSLs is the product under the optimum condition in shaker bath reactor.

TGA characteristic of different SLs

In this study, the Thermogravimetric (TGA) curves was used to determine the thermal degradation and thermal stability of each SLs. The TGA analysis of the MSLs, CSLs and SPOSLs are presented in Fig 2. The thermal decomposition of each sample takes place within the temperature range of a program, with a temperature range of 25 °C to 700°C. In table 3 the MSLs showed four stages of weight loss process, while there were three and two stages of CSLs and SPOSLs respectively. More stages suggested that a higher value of the thermal stability it will have [28]. As illustrated in Table S1, the degradation steps are in the temperature range of 27.25 °C - 154.8°C, 155.61 °C - 295.81°C, 295.81 °C - 484.29 °C and 485.22 °C - 694.22°C. The percentage of the loss weight composites at corresponding transition was 5.97, 72.80, 18.07 and 2.67%, respectively. Therefore, it can be concluded that the thermal stability of the MSLs had a higher value as compared to CSLs and SPOSLs.

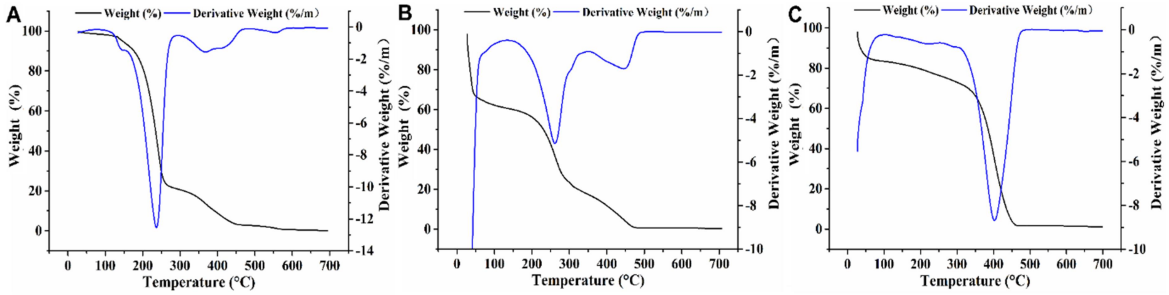

Fig. 2. Thermogravimetric map of SLs in different reactors. Condition: (A) TAG product in microwave reactor with substrate ratio 1:5 at 60 ℃ 50 W and under enzyme loading 7% (w/w) in 1 h; (B) TAG product in Shaker bath reactor with substrate ratio 1:5 at 60 ℃ and under enzyme loading was 7% in 3 h; (C) Original silkworm pupa oil.

The TAG profiles of SLs in different reactors

Chromatograms of the same concentration of four standard TAGs by HPLC-ELSD are shown in Fig. 3. Two products were diluted by the same fold. The abbreviation were listed in Table 4. Each kind of TAGs were identified with ECN of TAGs, the retention time of standard TAGs and the previous researches of the qualitative analysis of TAGs [30]. The retention time of TAGs increases with increasing of DBs and the equivalent carbon number (ECN) [31]. In Table 3S, palmitic and oleic, palmitoleic and linoleic have the same value of ECN (Table S2) so the sequence of TAGs were arely reported to the previous reports. The chromatograms of standard TAGs were shown in Figure 3C and the profiles of MSLs and CSLs were shown in Figs. 3A and B. These figure indicated that there are a lot of UFAs bind to triglyceride structures of MSLs. It is proved by the relative content of TAGs in Table 6. The relative content of LnLnLn, LLLn, LnOLn, OLL and OOO were 11.82%, 12.48%, 14.25%, 17.03% and 14.98%, respectively. While the relative content of these TAGs were 2.03%, 8.42%, 2.28%, 11.90% and 7.27%, respectively. In side of this relative content, 2 times increasing was gotten in OOO, moreover the relative content of OPO in MSLs were 6 times of TAGs in CSLs. And in the results of DSC MSLs got lower melting point than CSLs still proved that MSLs has better nutritional function and for UFAs supplements it has better characteristics. In other words, the transesterification reaction in microwave reactor is more conducive to unsaturated fatty acid binding on triglyceride structure.

Table 6 Relative content of MSLs and CSLs

TAG molecular species[a]	The relative content of SLs (%)	
	MSLs	CSLs
LnLnLn	11.82	2.03
LnLLn	4.90	3.65
LLLn	12.48	8.42
LnOLn	14.25	2.28
LnLnP	2.98	14.50
LLL	5.58	8.90
OLL	17.03	11.90
LLP	3.20	0.51
OOPo	1.77	7.04
OLP	1.65	1.27
OOO	14.98	7.27
OPO	5.63	0.93
SLnS	1.46	0.44
OLO	0	7.45
LnLP	1.48	16.84
SLnP	0.41	5.97
SLP	0.41	0.61

[a] TAG names do not illustrate positional location of fatty acids in the triacylglycerols.

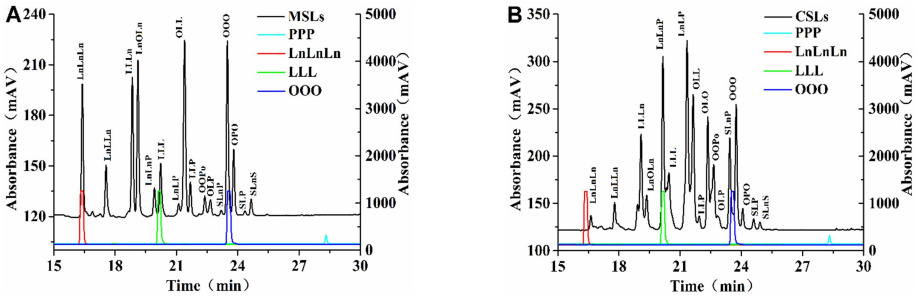

Fig. 3. The HPLC-ELSD chromatogram of MSLs (A) and CSLs (B). The ordinate on the left is the absorbance of the standard TAGs (PPP, LLL, OOO, LnLnLn). The ordinate on the right is the absorbance of the products (MSLs,CSLs).

The secondary structure of Lipozyme RM IM in different reactors

Fourier infrared spectroscopy (FT-IR) was used to detect the evolution of the secondary structure of Lipozyme RM IM in microwave and heat bath reactor. As showed in Fig. 4, the detection of the infrared spectra of enzymes in the 1,700-1,600 cm-1 region provided the quantitative and qualitative information on the secondary structure of the protein [32]. Peak fitting was performed on the amide I band of the Amidel band (1,700-1,600 cm^{-1}) using Gaussian-shaped components. Table S3 shows that after the microwave reaction the content of the β-sheet in enzyme was increased to 27.48% while the primary enzyme did not have this secondary structure and aggrandize of the Random (0.35%) was also been found. On the other hand, the content of the *β*-turn descended from 99.45% to 59.25%. Petroleum ether was used to washing the enzyme after reaction and could not lead to this change. The secondary structure of Lipozyme RM IM in shaker bath reactor also has been changed but different from the enzyme in microwave irradiation. After been used in shaker bath reactor, the content of the *β*-sheet in enzyme was increased to 10.25% and the increase of Random (2.22%) was also been found. But the difference was the content of α-helix, after reaction in microwave reactor the increase of *α*-helix (0.66%) was found and there was no *α*-helix in enzyme after used in shaker bath. The loss of *α*-helix structure inferred correlation in the enzyme activity [33]. The loss of *α*-helix might be produced by higher relative content of OOO-style in the products under microwave irradiation. Therefor the microwave irradiation could have the influence on the activity of Lipozyme RM IM.

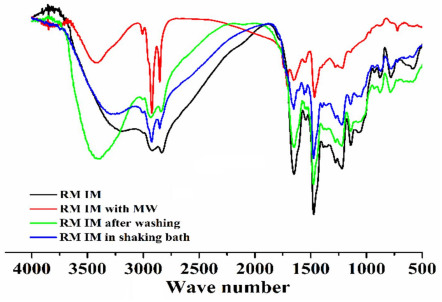

Fig. 4. Fourier infrared spectroscopy of Lipozyme RM IM in different reactors. RM IM without MW represents for the unused Lipozyme RM IM. RM IM with MW represent for the Lipozyme RM IM which was used under the optimum condition in microwave reactor; RM IM after washing represent for the Lipozyme RM IM washed with petroleum ether; RM IM in shaker bath represent for the Lipozyme RM IM which was used under the optimum condition in shaker bath.

Conclusions

Novel SLs with better thermal stability, lower melting temperature, lower peroxide value and enriched with unsaturated fatty acids were produced by enzymatic acidolysis from silkworm pupa oil using OA under the microwave irradiation. Products contained larger amounts of unsaturated fatty acids (71.65%) at *sn*-1,3 position. However, the saturated fatty acids content decreased from 34.43% to 28.35%. OOO-style and OPO-style was increased 2.06-fold and 6-fold respectively compared with that in a shaker bath reactor. The MSLs synthesized using the solvent-free system under the microwave irradiation developed in this study may be more suitable for direct human consumption and promote the utilization of silkworm pupa. The microwave-assisted contribute to enrich the UFAs such as ALA and LA and bring the UFAs of SPO into being polyunsaturated fatty acid glycerides. One hours was taken to enzymatic synthesis of structure lipids enriched unsaturated fatty acids from silkworm pupae oil under microwave irradiation.

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The authors have declared no conflicts of interest.

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Electronic Supplementary Material

Table S1 Results of TGA analysis different SLs.

Sample	NO. of transition	Transition temperature (℃)		Weight loss at transition (%)
		T_i	T_f	
MSPOSLs[a]	1	27.25	154.68	5.97
	2	155.61	295.81	72.80
	3	295.81	484.29	18.07
	4	485.22	694.22	2.67
CSPOSLs[b]	1	26.98	143.09	37.54
	2	144.04	342.42	42.13
	3	343.37	706.13	17.68
SPOSLs[c]	1	27.44	106.64	14.70
	2	107.57	697.87	82.16

[a] Fatty acids composition of silkworm pupae oil interesterified product SLs under the optimum condition in microwave reactor.
[b] Fatty acids composition of silkworm pupae oil interesterified product SLs under the shaking bath reactor. Conditions, Substrate ratio, 1:5; Temperature, 60 °C; Enzyme loading, 7%; Time, 3 h.
[c] The silkworm pupa oil extracted from silkworm pupa.

Table S2 Trivial names of fatty acids found in triacylglycerols of this studied silkworm pupa oil listed with their addreviation, carbon numbers (CN), double bond numbers (DB) and equivalent carbon numbers (ECN)

Trivial names	Abbreviation	CN:DB	ECN
Palmitic	P	C16:0	16
Palmitoleic	Po	C16:1	14
Stearic	S	C18:0	18
Oleic	O	C18:1	16
Linoleic	L	C18:2	14
Linolenic	Ln	C18:3	12

Table S3 FT-IR was used to detect the secondary structure of the Lipozyme RM IM with microwave reactor and without microwave reactor.

Sample	Secondary structure type and content (%)			
	Random	α-helix	β-turn	β-sheet
RM IM [a]	-	0.55	99.45	-
RM IM in MW [b]	0.35	0.66	59.25	27.48
RM IM in shaking bath [c]	2.22	-	87.53	10.25
RM IM after washing [d]	-	0.38	99.62	-

[a] Original Lipozyme RM IM.
[b] The Lipozyme RM IM was used in the microwave reactor.
[c] The Lipozyme RM IM was used in shaking bath reactor.
[d] The Lipozyme RM IM washed with petroleum ether.