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Lipase-Catalyzed Preparation of Human Milk Fat Substitutes from Palm Stearin in a Solvent-Free System

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ABSTRACT: Human milk fat substitutes (HMFSs) were synthesized by lipozyme RM IM-catalyzed acidolysis of chemically interesterified palm stearin (mp = 58 °C) with mixed FAs from rapeseed oil, sunflower oil, palm kernel oil, stearic acid, and myristic acid in a solvent-free system. Response surface methodology (RSM) was used to model and optimize the reactions, and the factors chosen were reaction time, temperature, substrate molar ratio, and enzyme load. The optimal conditions generated from the models were as follows: reaction time, 3.4 h; temperature, 57 °C; substrate molar ratio, 14.6 mol/mol; and enzyme load, 10.7 wt % (by the weight of total substrates). Under these conditions, the contents of palmitic acid (PA) and PA at sn-2 position (sn-2 PA) were 29.7 and 62.8%, respectively, and other observed FAs were all within the range of FAs of HMF. The product was evaluated by the cited model, and a high score (85.8) was obtained, which indicated a high degree of similarity of the product to HMF.

KEYWORDS: Human milk fat substitutes, lipozyme RM IM, acidolysis, palm stearin, response surface methodology, solvent-free system

INTRODUCTION

Structured lipids (SLs) are referred to triacylglycerols (TGs) that are chemically or enzymatically modified to meet special functions for nutrition, food, and/or pharmaceutical applications by changing the position and/or composition of fatty acids (FAs) in the glycerol backbone.[1,2] Enzymatic modification of TGs, due to its efficacy under mild reaction conditions and positional specificity, has been widely used in the production of SLs, and lipozyme RM IM (RML from *Rhizomucor miehei* immobilized on Duolite ES 562), a commercially available enzyme with high activity and good stability, is a sn-1,3-specific lipase commonly used in those enzymatic reactions.[3,4] Up until now, typical SLs produced by lipozyme RM IM are human milk fat substitutes (HMFSs), modified fish oil products, and other lipid products.[5−7]

Human milk, which can provide necessary nutrients and other physiologically active substances, is considered the best food for infants. Human milk contains 3−5% total lipids (TGs > 98%), which is the main source of energy (>50%).[8] FA composition of lipids in human milk varies with such factors as lactation stage, mother's diet, season, genetics, and individual conditions.[9,10] However, some common characteristics can also be observed as follows: (i) The major FA in human milk fat (HMF) is oleic acid (25−35%), followed by palmitic acid (20−30%), linoleic acid (10−20%), stearic acid (5−9%), myristic acid (4−9%), and lauric acid (3−7%); (ii) 60−70% of palmitic acid is at the sn-2 position; (iii) oleic acid, linoleic acid, and other unsaturated FAs are mainly distributed at sn-1,3 positions. The structure of HMF with most of the saturated FAs at sn-2 position and unsaturated FAs at sn-1,3 positions, which is different from other oils and fats (except lard), has substantial influence on the absorption of TGs in infants. The TGs were selectively hydrolyzed to sn-2 monoacylglyerols (sn-2 MAGs) and FAs by pancreatic lipase. The sn-2 MAGs can be efficiently absorbed in small intestine,[11] whereas the absorption efficiency of the FAs from sn-1,3 positions depends on the length of carbon chain and degree of unsaturation.[12] Long-chain saturated FAs, which can form poorly absorbed soaps, result in loss of energy and calcium.[13,14] Because of the formation of these soaps, stool hardness, constipation, and, in some times, obstructions may occur.[15] Therefore, the unique structure of human milk fat is beneficial for absorption of palmitic acid and calcium and has a great function in the development of infants.

Sometimes, mothers cannot provide breast feeding for their babies due to medical, metabolic, and economical reasons, and an alternative formula is needed. Therefore, many studies have focused on the production of HMFSs by using different starting materials (lard and tripalmitin), lipases (lipozyme RM IM and lipozyme TL IM), and enzymatic reactors (batched reactor and packed bed reactor).[16−20]

Palm stearin (mp = 58 °C), which is a fraction of palm oil and widely used in specialty fats, because of its proper content of palmitic acid (70.1%), can be used as the starting material after interesterification to produce HMFSs by acidolysis. In the acidolysis reaction, many factors such as reaction time, temperature, substrate molar ratio, and enzyme and water contents have influence on the final yields of SLs, of which different enzymes can give different results, even different immobilized preparations of the same enzyme.[21,22] In this study, the objective was to produce HMFSs by acidolysis of interesterified palm stearin with mixed FAs catalyzed by lipozyme RM IM. Response surface methodology (RSM) was used to analyze the effects of selected variables including reaction time, temperature, substrate molar ratio, and enzyme load and their interactions on the responses including the content of palmitic acid (PA) and PA at the sn-2 position (sn-2 PA) and to determine the optimum

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Table 1. FA Composition of Rapeseed Oil, Sunflower Oil, and Palm Stearin

	palm kernel oil	rapeseed oil	sunflower oil	palm stearin		
FA[a] (mol %)	total	total	total	total	sn-2[b]	sn-2[c]
C8:0	4.4					
C10:0	4.8					
C12:0	55.7			0.7	0.1	0.5
C14:0	15.1			1.3	0.9	1.0
C16:0	6.4	4.4	5.7	70.1	56.8	69.8
C18:0	1.1	0.9	5.6	5.2	2.9	5.0
C18:1n-9	10.2	63.7	26.5	18.7	30.9	19.3
C18:2n-6	2.1	21.0	61.7	3.9	8.3	4.3
C18:3n-3		9.9	0.4			

[a] C8:0, caprylic; C10:0, capric; C16:0, palmitic; C18:0, stearic; C18:1n-9, oleic; C18:2n-6, linoleic acid; and C18:3n-3, linolenic acid. [b] Indicates FA composition at the sn-2 position before chemical interesterification. [c] Indicates FA composition at the sn-2 position after chemical interesterification. The content of lower glycerides of interesterified palm stearin was 2.8% after alcohol washing.

conditions for the production. The degree of similarity of the product to HMF was also evaluated by the related model.

MATERIALS AND METHODS

Materials. Lipozyme RM IM, a sn-1,3-specific lipase from *R. miehei*,[5] was purchased from Novozymes A/S (Bagsvaerd, Denmark). Pancreatin (Porcine pancreas) powder was bought from Sigma Corp. (United States). Stearic and myristic acids (purity >98%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Palm stearin (mp = 58 °C) was provided by Jinguang Oil Co., Ltd. (Ningbo, China), and rapeseed oil, sunflower oil, and palm kernel oil were donated by Shanghai Kerry Oils & Grains Industries Co., Ltd. (Shanghai, China). The FA composition of palm stearin, rapeseed oil, sunflower oil, and palm kernel oil analyzed by GC was shown in Table 1. Silicic acid 60G TLC plates (10 cm × 20 cm) were purchased from Shanghai Shangbang Co., Ltd. (Shanghai, China). Methanol and glacial acetic acid were of high-performance liquid chromatograph (HPLC) purity. Hexane and ethyl ether were of analytical grade.

Preparation of FAs. FAs were prepared from rapeseed oil, sunflower seed oil, and palm kernel oil as described by Senenayeke and Shahidi.[23] Twenty-five grams of oil was saponified by a mixture of KOH (5.75 g), water (11 mL), and 95% (v/v) aqueous ethanol (66 mL) for 1 h at 60 °C by refluxing. Distilled water (50 mL) and hexane (2 × 100 mL) were then added to the mixture. The organic layer with unsaponifiable matters was discarded, and the aqueous layer containing the saponifiable matters was acidified with 3 M HCl to a pH of 1.0. The FAs were liberated after acidification and then extracted with hexane (50 mL). The hexane layer was dried over anhydrous sodium sulfate, and the FAs were recovered by removing the solvent with a rotary evaporator at 40 °C.

Chemical Interesterification. Palm stearin, after dried at 105 °C for 90 min under vacuum (1.33×10^3 Pa), was mixed with 0.5% sodium methoxide powder and interesterified for 60 min at 80 °C with magnetic stirring. It was then cooled to 70 °C, and 5% aqueous citric acid (20%) was added to inactivate the catalyst.[24] After 15 min of stirring, the mixture was washed with warm water five times. The interesterified fat was then bleached with 1% of bleaching earth for 30 min at 90 °C under vacuum, followed by filtration through a Whatman no. 4 filter paper. Finally, the interesterified palm stearin was washed with 95% ethanol (w/v = 1:2) three times at 60 °C to remove the produced lower glycerides in the material. The time course of the interesterification reaction (sn-2 PA as the index) was given in Figure 1, and the FA composition of sn-2 position of palm stearin before and after interesterification is also shown in Table 1.

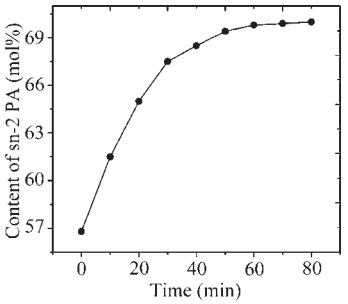

Figure 1. Time course of chemical interesterification of palm stearin. Reaction conditions: temperature, 80 °C; catalyst load, 0.5%; and vacuum, 1.33×10^3 Pa.

Enzymatic Acidolysis. Palm stearin (2 g) was mixed with mixed FAs at different substrate molar ratios ranging from 10 to 16 in 25 mL round-bottom flasks, and then, the lipase (6−14 wt %, by the weight of total substrates) with water content at 3.5 wt % (by the weight of enzyme) was added to start the reaction. Samples were flushed with nitrogen and incubated in a water bath with magnetic agitation at 250 rpm and 50−70 °C.

HPLC Analysis of Lower Glycerides. The lower glycerides, including MAGs and diacylglyerols (DAGs), were analyzed by a waters 2420 HPLC system, equipped with an evaporative light-scattering detector (ELSD). The ELSD was set at 60 °C at a nitrogen nebulizer gas pressure of 30 psi and a gain of 5 for a normal phase system. The separation was carried out on a Spherisorb Silica column (5 μm, 4.6 mm × 250 mm; Waters Corp., Milford, MA) eluted with a binary gradient of solvent A (hexane/isopropanol = 99/1, v/v) and solvent B (hexane/isopropanol/acetic acid = 1/1/0.01, v/v/v). A linear gradient of solvent A from 100 to 80% over 10 min was applied at a flow rate of 1 mL/min, then to 70% A in 4 min, followed to 100% A in 5 min, and then kept at 100% A for 5 min. The content of the lower glycerides was calculated based on their area percentage.

Experimental Design. A three-level, four-factor Box−Behken design was employed, in which 29 experiments were required, to optimize the acidolysis reactions. The factors and their levels selected were substrate molar ratio (12, 14, and 16 mol/mol; FAs/palm stearin), temperature (55, 60, and 65 °C), enzyme load (8, 10, and 12 wt %; by the weight of total substrates), reaction time (2, 3, and 4 h). The independent factors and levels in both coded and uncoded parameters and experimental design with observed values for the responses are presented in Table 2. Experiments were run randomly, and duplicate reactions were carried out at all design points.

Triglyceride Isolation. A 50 μL aliquot of the reaction product was taken out from the reaction system and isolated by thin-layer chromatography (TLC) plates with hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol) as the developing solvent.[25] The plates were then sprayed with 0.2% 2,7-dichlorofluorescein in methanol and visualized under UV light. The band corresponding to TAG was scraped off and methylated with 3 mL of 4% H_2SO_4 in methanol at 90 °C for 20 min under nitrogen. The FA methyl esters were extracted twice with 2 mL of hexane, dried over an anhydrous sodium sulfate column, and concentrated using nitrogen.

FA Composition Analysis. The FA composition of the product was analyzed with a GC-14B gas chromatograph, equipped with a flame ionization detector (Shimadzu, Tokyo, Japan) and a fused-silica capillary column (PEG-20M, 30 m × 0.32 mm × 0.5 μm). The column was initially held at 100 °C for 4 min, followed by temperature programming to 180 °C at the rate of 15 °C/min, and then held at 180 °C for 4 min and

Table 2. Experimental Design and Results of Content of PA (Y_1) and sn-2 PA (Y_2)

treatment[a]	reaction time	reaction temperature	enzyme load[b]	substrate molar ratio	content of PA	content of sn-2 PA
number	X_1 (h)	X_2 (°C)	X_4 (wt %)	X_3 (mol/mol)	Y_1 (mol %)	Y_2 (mol %)
1	0 (3)[c]	0 (60)	−1 (8)	−1 (12)	35.1	65.4
2	0 (3)	1 (65)	−1 (8)	0 (14)	32.6	60.2
3	0 (3)	0 (60)	1 (12)	1 (16)	28.7	59.5
4	1 (4)	1 (65)	0 (10)	0 (14)	30.3	56.7
5	0 (3)	0 (60)	0 (10)	0 (14)	31.8	62.3
6	−1 (2)	1 (65)	0 (10)	0 (14)	32.0	63.5
7	0 (3)	1 (65)	0 (10)	1 (16)	28.9	57.6
8	−1 (2)	0 (60)	0 (10)	−1 (12)	34.6	65.8
9	1 (4)	0 (60)	−1 (8)	0 (14)	31.7	61.4
10	0 (3)	0 (60)	0 (10)	0 (14)	32.2	62.8
11	0 (3)	0 (60)	0 (10)	0 (14)	31.3	61.6
12	−1 (2)	0 (60)	−1 (8)	0 (14)	36.6	66.1
13	0 (3)	0 (60)	1 (12)	−1 (12)	32.2	62.5
14	−1 (2)	0 (60)	1 (12)	0 (14)	32.1	65.3
15	0 (3)	0 (60)	0 (10)	0 (14)	31.5	62.4
16	1 (4)	0 (60)	1 (12)	0 (14)	29.8	58.2
17	1 (4)	0 (60)	0 (10)	−1 (12)	33.1	61.7
18	−1 (2)	0 (60)	0 (10)	1 (16)	32.5	64.8
19	1 (4)	−1 (55)	0 (10)	0 (14)	31.6	63.2
20	0 (3)	−1 (55)	1 (12)	0 (14)	32.4	63.7
21	−1 (2)	−1 (55)	0 (10)	0 (14)	36.3	67.3
22	0 (3)	−1 (55)	0 (10)	−1 (12)	35.5	65.5
23	1 (4)	0 (55)	0 (10)	1 (16)	28.5	58.1
24	0 (3)	−1 (55)	0 (10)	1 (16)	29.2	64.3
25	0 (3)	0 (55)	0 (10)	0 (14)	31.2	62.5
26	0 (3)	0 (55)	−1 (8)	1 (16)	30.6	61.3
27	0 (3)	−1 (55)	−1 (8)	0 (14)	33.2	66.7
28	0 (3)	1 (65)	1 (12)	0 (14)	30.4	57.5
29	0 (3)	1 (65)	0 (10)	−1 (12)	32.4	61.3

[a] Treatments were run in random order. [b] Enzyme load (wt %, by the weight of total substrates). [c] Numbers in parentheses represent actual experimental amounts.

to 215 °C at the rate of 4 °C/min. The injection port and detector temperatures were both set at 250 °C. The FA methyl esters were identified by comparing retention time with the standards, and the relative contents expressed as mol % were then calculated.

Pancreatic Lipase Hydrolysis. Hydrolysis of TAG obtained after TLC isolation was carried out according to the method described by Luddy et al.[26] One milliliter of 1 M Tris-HCl buffer (pH 8.0), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 20 mg of pancreatic lipase were added to the TAG. The mixture was incubated in a water bath at 40 °C for 3 min with vigorous shaking, and then, 1 mL of 6 M HCl and 2 mL of diethyl ether were added and centrifuged. Diethyl ether was dried by anhydrous sodium sulfate and evaporated by nitrogen to 200 μL. The hydrolytic products were separated on silica gel G TLC plates, and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The band corresponding to sn-2 MAGs was scraped off, methylated, and analyzed as mentioned above.

Statistical Analysis. The experimental data from the Box−Behken design were analyzed by RSM with Design Expert software (version 7.1.3.1, State-Ease, Inc., Statistics Made Easy, Minneapolis, MN). Second-order coefficients of the model were generated by multiple regressions with backward elimination, and the statistical significance and the fitting quality of the model were evaluated by the coefficients of determination (R^2) and analysis of variance (ANOVA). The quadratic polynomial equation employed to fit the data is shown below.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is one of the two responses, X_i and X_j are the coded independent variables, β_0 is the intercept, β_i is the linear term coefficient, β_{ii} is the quadratic term coefficient, and β_{ij} is the cross-term coefficient.

Evaluation of HMFSs. Different HMFSs have different FA compositions and position distributions. Therefore, it is necessary to evaluate the quality of HMFSs by using some evaluation criteria. A model established by Wang et al.[27] was used in this experiment to evaluate the degree of similarity of the produced HMFSs to human milk TGs. On the basis of two equally important elements that determine the chemical structure of human milk TGs, that is, total and sn-2 FA composition, a "deducting score" principle was used in the model to evaluate the degree of similarity.[27] The model was then given as follows:

$$G = G_1 + G_2 \quad (2)$$

$$G_1 = 50 \sum_{i=1}^{n} \left(\frac{|B_i - A_i|}{A_i} \frac{D_i}{\sum_{i=1}^{n} D_i} \right) \quad (3)$$

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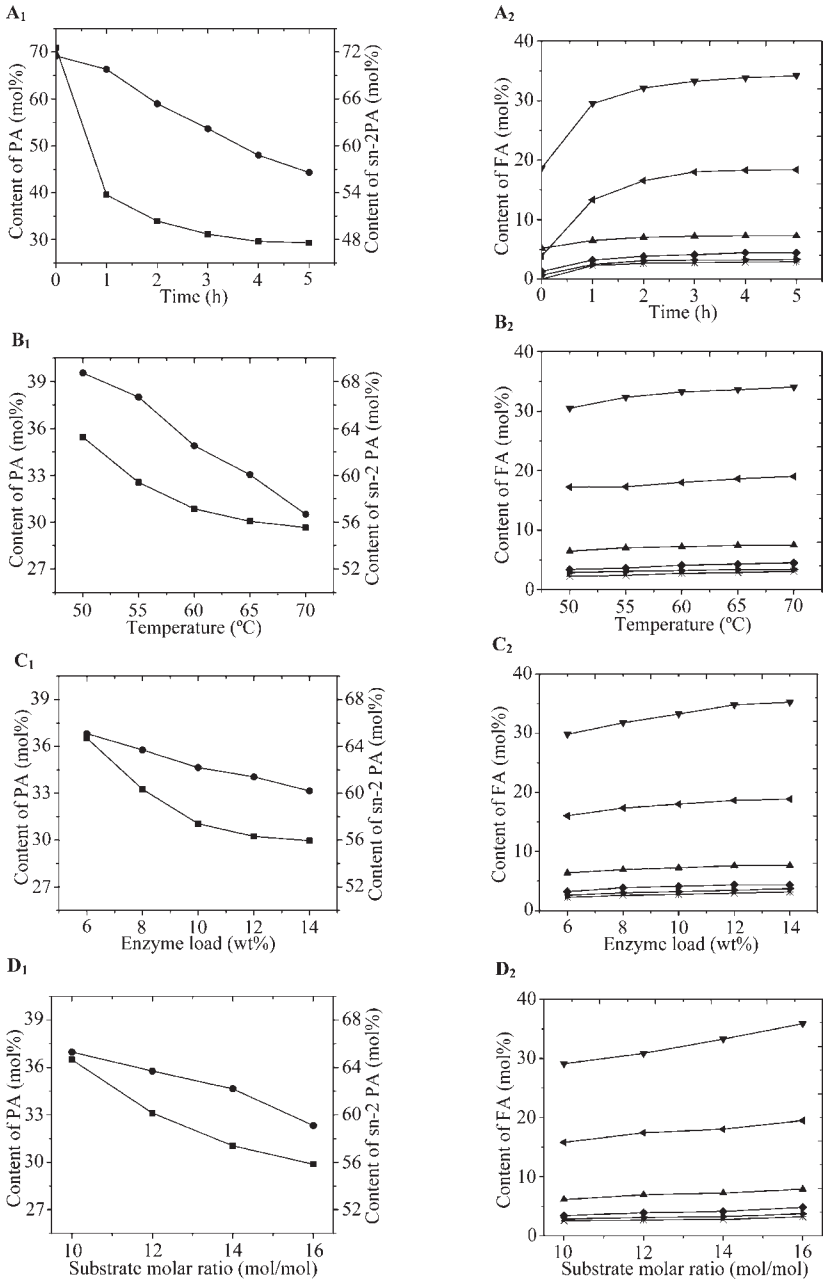

Figure 2. Effects of reaction time, temperature, substrate molar ratio, and enzyme load on the content of the observed FAs. Palmitic acid (■), sn-2 palmitic acid (●), oleic acid (▼), linoleic acid (left-pointing triangle), stearic acid (▲), myristic acid (♦), lauric acid (right-pointing triangle), and linolenic acid (∗). (A_1), (A_2): 60 °C; substrate molar ratio, 14 mol/mol (FAs/palm stearin); enzyme load, 10 wt % (by the weight of total substrates). (B_1), (B_2): substrate molar ratio, 14 mol/mol; enzyme load, 10 wt %; 3 h. (C_1), (C_2): 60 °C; enzyme load, 10 wt %; 3 h, (D_1), (D_2): 60 °C; substrate molar ratio, 14 mol/mol; 3 h.

$$G_2 = 50\sum_{i=1}^{n}\left(\frac{|B_{i(\text{sn-2})} - A_{i(\text{sn-2})}|}{A_{i(\text{sn-2})}}\frac{D_{i(\text{sn-2})}}{\sum_{i=1}^{n} D_{i(\text{sn-2})}}\right) \quad (4)$$

where G is the score that the evaluated TGs gets and the maximum score for G is 100; G_1 and G_2 are the scores that the total FAs and sn-2 FAs get; B_i and $B^{i(\text{sn-2})}$ are the content of different FAs in the total FAs and sn-2 FAs; A_i and $A^{i(\text{sn-2})}$ are the lower or the upper limit of 95% reference range of total FAs and sn-2 FAs, and the values of lower or upper limits were obtained from HMF. When B is higher than the upper limits of corresponding FA content, A is selected as the upper limit, and vice versa. If B is within the range, $|B_i - A_i|/A_i$ and $|B_{i(\text{sn-2})} - A_{i(\text{sn-2})}|/A_{i(\text{sn-2})}$ are kept at zero. D_i and $D^{i(\text{sn-2})}$ are mean values of content of total FAs and sn-2 FAs of HMF.

RESULTS AND DISCUSSION

Selection of the Starting Materials and Acyl Donors. On the basis of the FA composition and position distribution of HMF, the target of this study was to synthesize SLs with less than 30% total PA and more than 60% sn-2 PA and other FAs including lauric acid, myristic acid, stearic acid, oleic acid, linoleic

acid, and linolenic acid within the range of the corresponding FAs in HMF.

Palm stearin (mp = 58 °C, PA = 70.1%), after chemical interesterification, was used as the starting TGs for its suitable PA content. The FA composition and distribution of palm stearin before and after interesterification are shown in Table 1. As compared with HMF, palm stearin contains more palmitic acid, less lauric acid, myristic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid. The ratio of linoleic acid to linolenic acid in palm stearin is around 44.1, which is markedly different from that of HMF (5−15), reported by Yang et al.[4] Although some disadvantages exist, the interesterified palm stearin has about 70% PA at the sn-2 position, which is a great advantage over other oils. On the basis of this characteristic, it is possible to prepare SLs with similar FA composition and distribution to HMF by acidolysis, the interesterified palm stearin with mixed FAs catalyzed by lipozyme RM IM.

In preliminary experiments, the FAs of rapeseed oil, sunflower oil, and palm kernel oil that were rich in oleic acid (63.7%), linoleic acid (61.7%), and lauric acid (55.7%), respectively, and stearic acid and myristic acid were mixed together at different molar ratios as acyl donors to react with the interesterified palm stearin catalyzed by lipozyme RM IM. It was found that when the ratio was 2:1:0.5:0.2:0.3 (rapeseed oil FAs/sunflower oil FAs/palm kernel oil FAs/stearic acid/myristic acid), that is, the composition of mixed FAs was as follows: lauric acid (6.9%), myristic acid (9.3%), palmitic acid (4.6%), stearic acid (7.7%), oleic acid (39.7%), linoleic acid (26.4%), and linolenic acid (5.1%); the FA composition and position distribution of the obtained SLs were similar to these of HMF. Therefore, the mixed FAs at the molar ratio of 2:1:0.5:0.2:0.3 were selected as the acyl donors in the latter experiments.

Selection of Independent Variables and Their Levels. Lipase-catalyzed acidolysis is a two-step reaction involving initial hydrolysis and subsequent re-esterification, in which hydrolysis is the rate-controlling step.[28] DAGs are considered as the intermediates in the reaction, which are produced by hydrolysis from original TGs and re-esterified with FAs to form new TGs. However, DAGs are also the precursor of side reactions (acyl migration) since they are not thermodynamically stable,[29] which lead to a nonspecificity of the lipases and the formation of byproduct. Therefore, factors, including temperature, water content, acyl donors, and reaction time, etc., which have an influence on the formation of DAGs or occurrence of acyl migration, affect the purity of the product.[30] Meanwhile, enzymatic acidolysis is also a reversible reaction, in which the product yield under reaction equilibrium is decided by the substrate ratio, that is, the ratio between FAs and TGs, and the rate to reach the equilibrium is related to many factors such as reaction temperature, enzyme load, and water content.[31] On the basis of the above-mentioned reasons, it is necessary to optimize reaction conditions to increase the efficiency of the reaction and decrease the formation of byproduct. In preliminary experiments, the increase of water content of the enzyme was found to have little influence on FA composition of the final product in this system. Meanwhile, it decreased the content of sn-2 PA and increased the content of lower glycerides, which were not expected in the experiment. Therefore, the water content of the enzyme was fixed at the initial value, and the effects of reaction time, temperature, enzyme load, and substrate molar ratio were investigated in the experiment.

The effects of different independent variables on the content of PA, sn-2 PA and other FAs including lauric acid, myristic acid, stearic acid, oleic acid, linoleic acid, and linolenic acid are shown in Figure 2, respectively. As the substrate molar ratio increased, the content of PA showed decreasing patterns, whereas the content of PA showed decreasing flat patterns with other variables increasing. The content of sn-2 PA decreased as the four variables increased, whereas the decrease rate was fastest with the temperature increasing. Other investigated indexes including content of lauric acid, stearic acid, oleic acid, linoleic acid, and linolenic acid were all within the range of the corresponding FAs in HMF after 3 h of reaction. Therefore, on the basis of the above experimental results, the contents of PA and sn-2 PA were selected as the dominating indexes, and the contents of other FAs were chosen as the assistant indexes, which were detected but not reported in the optimization experiments. Meanwhile, to meet the requirements of the targeted content of PA (<30%) and sn-2 PA (>60%), the lower, middle, and upper levels of the four independent variables were chosen in Table 2 considering the interactions of different variables.

Table 3. Regression Coefficients and Significance Values (*P* Values) of the Second-Order Polynomials after Backward Elimination

	content of PA (mol %)		content of sn-2 PA (mol %)	
variable	coefficient	*P* value	coefficient	*P* value
intercept	31.72	<0.0001	62.24	<0.0001
X_1	−1.61	<0.0001	−2.71	<0.0001
X_2	−0.97	<0.0001	−3.02	<0.0001
X_3	−1.18	<0.0001	−1.20	<0.0001
X_4	−2.06	<0.0001	−1.78	<0.0001
X_1X_2	0.75	0.0224	−0.68	0.0062
X_1X_3	0.65	0.0442	−0.60	0.0130
X_1X_4	−0.67	0.0374	−1.40	<0.0001
X_2X_4	0.70	0.0316	−1.20	<0.0001
X_1X_1	0.69	0.0068	0.62	0.0015

Model Fitting. The best-fitting quadratic models for the content of PA and sn-2 PA were obtained after multiple regression and backward elimination. The model coefficients and *p* values for the responses were statistically calculated based on experimental results (Table 2) and are shown in Table 3.

It can be seen from Table 3 that all first-order parameters of both responses (content of PA and sn-2 PA) were significant with *p* values less than 0.0001. In the second-order parameters, time × time with positive effects was the only significant parameter for both responses. The interaction terms time × temperature, time × enzyme load, time × substrate molar ratio, and enzyme load × substrate molar ratio were found to be significant for both responses. The difference was that, for the content of PA, only time × substrate molar ratio had negative effects; however, for the content of sn-2 PA, all interaction terms affected negatively.

According to ANOVA presented in Table 4, the models with *p* values less than 0.0001 were statistically significant and highly appropriate for the prediction. *p* values of lack of fit for two models were 0.2153 and 0.2872, respectively, which indicated that the models were stable and adequate to predict the variation of the responses.[32] Further analysis by comparison between the predicted and the experimental values showed the predicted values correlated well in linearity with the observed ones (Figure 3; $R_1^2 = 0.9631$, $R_2^2 = 0.9896$, respectively). The result also indicated that the generated models adequately

Table 4. ANOVA for Response Surface Quadratic Models

source	sum of squares	degree of freedom	mean square	*F* value	*P* value
		total PA			
model	123.17	14	8.80	26.10	<0.0001
residual	4.72	14	0.34		
lack of fit	4.06	10	0.41	2.46	0.1999
pure error	0.66	4	0.17		
total	127.89	28			
CV % = 1.81, R^2 = 0.9631, and R^2_{adj} = 0.9262					
		sn-2 PA			
model	273.36	14	19.53	95.01	<0.0001
residual	2.88	14	0.21		
lack of fit	2.37	10	0.24	1.17	0.2872
pure error	0.51	4	0.13		
total	276.24	28			
CV % = 0.73, R^2 = 0.9896, and R^2_{adj} = 0.9792					

represented the relationship between the responses and the tested parameters.

The two quadratic polynomial model equations for the content of PA (mol %) and sn-2 PA (mol %) can therefore be written as follows:

$$Y_1\ (\text{mol}\%) = 31.72 - 1.61X_1 - 0.97X_2 - 1.18X_3 - 2.06X_4 + 0.75X_1X_2 + 0.65X_1X_3 - 0.67X_1X_4 + 0.70X_2X_4 + 0.69{X_1}^2 \quad (5)$$

$$Y_2\ (\text{mol}\%) = 62.24 - 2.71X_1 - 3.02X_2 - 1.20X_3 - 1.78X_4 - 0.68X_1X_2 - 0.60X_1X_3 - 1.40X_1X_4 - 1.20X_2X_4 + 0.62{X_1}^2 \quad (6)$$

where Y_1 and Y_2 are the predicted values for the content of PA and sn-2 PA, respectively, and X_1, X_2, X_3, and X_4 are the coded variables as described in Table 2.

Optimization of the Reaction. The relationship between the independent variables and the responses can be better understood by examining contour plots given in Figures 4 and 5, which were obtained by interactions of variables on content of PA and sn-2 PA. Medium levels were used for the other two variables when the contour plots were drawn.

The interactions of reaction time with temperature, of enzyme load with reaction time, of reaction time with substrate molar ratio, and of substrate molar ratio with temperature on the content of PA are shown in Figure 4A–D, respectively. As can be seen from Figure 4A, a longer reaction time resulted in lower content of PA, but the decrease rate became smoother as the reaction time exceeded 3.5 h. When the temperature was fixed at 60 °C, increasing the reaction time from 3 to 3.5 h gave a decrease of 1%, whereas increasing the reaction time from 3.5 to 4 h only gave a decrease of 0.4%, in the content of PA. Similarly, a higher temperature also resulted in a lower content of PA, which was due to the reduction of mass transfer limitations and making the substrate more available to the enzyme.[33] However, high temperature would increase the denaturation rate of enzyme and the probability of occurrence of side effects such as acyl migration. Therefore, it was necessary to keep the temperature in a suitable range. In Figure 4B, a longer reaction time and higher enzyme load resulted in a lower content of PA. The reaction rate increased with the enzyme load increasing, which decreased the time for equilibrium and improved the production efficiency. In Figure 4C,D, the content of PA increased as reaction time and substrate ratio increased; the substrate ratio and enzyme load increased in the observed ranges.

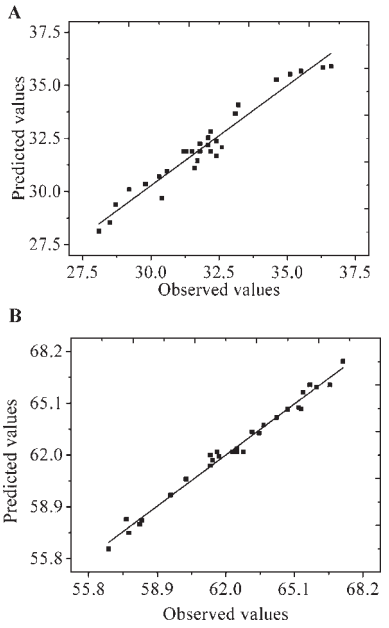

Figure 3. Relationship between the observed and the predicted values: (A) content of PA, with R^2 = 0.9631; (B) content of sn-2 PA, with R^2 = 0. 9896.

The interactions of reaction time with temperature, of enzyme load with reaction time, of reaction time with substrate molar ratio, and of substrate molar ratio with temperature on the content of sn-2 PA are shown in Figure 5A–D, respectively. All plots in Figure 5 give similar relationships with respect to each variable. As can be seen from Figure 5A, the content of PA at sn-2 position decreased with the increase of the reaction time and temperature. When the reaction time was fixed at 3 h, the content of sn-2 PA decreased from 65 to 62% as the temperature increased from 55 to 65 °C. The decrease of the content of sn-2 PA was due to the occurrence of acyl migration during acidolysis reaction. Acyl migration was a thermodynamic process following the general rule of Arrhenius law,[30] which indicated that the acyl migration rate was faster at a higher temperature. Therefore, a relative lower temperature to suppress acyl migration would be better for optimizing reaction conditions. In Figure 5B, the content of sn-2 PA decreased with the increase of the reaction time and enzyme load. However, the decrease rate was much faster with the reaction time increasing than that with the enzyme load increasing. The decrease in content of sn-2 PA with enzyme load increasing was probably due to more diacylglycerol produced with more enzyme loaded in the system, which was a key factor to induce acyl migration.[34] In Figure 5C, the content of sn-2 PA decreased as the substrate molar ratio increased, and the decrease rate was faster when the substrate molar ratio was at its higher levels. At the same time, high amounts of FAs used would be a limitation for industrial production of HMFSs, because many efforts were needed to

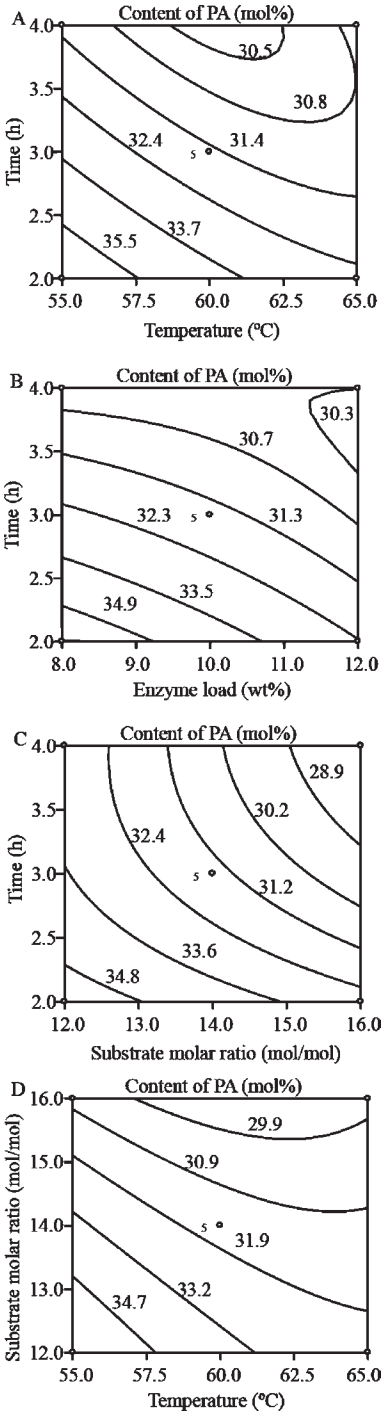

Figure 4. Response contour plots between two parameters for the content of PA: (A) reaction time vs temperature, (B) reaction time vs enzyme load, (C) reaction time vs substrate molar ratio, and (D) substrate molar ratio vs temperature.

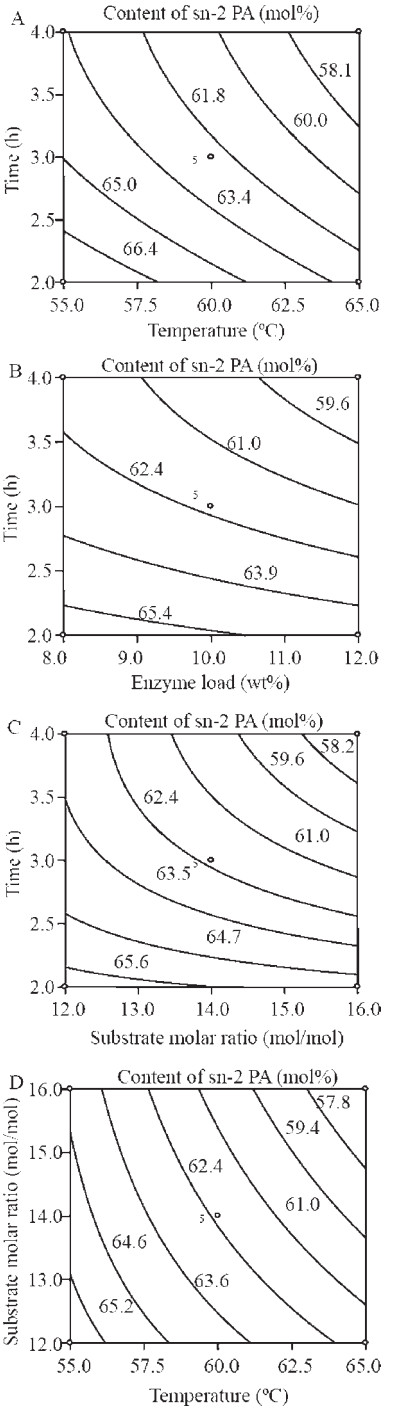

Figure 5. Response contour plots between two parameters for the content of sn-2 PA: (A) reaction time vs temperature, (B) reaction time vs enzyme load, (C) reaction time vs substrate molar ratio, and (D) substrate molar ratio vs temperature.

remove them to obtain purified HMFSs. Therefore, a suitable substrate molar ratio should be carefully determined by considering the indexes of HMFSs production, including the content of PA and sn-2 PA and the cost for the substrate and the purification process. In Figure 5D, the content of sn-2 PA increased with the substrate ratio and temperature increasing within the observed ranges.

The conditions for the targeted content of PA (<30%) and sn-2 PA (>60%) were generated by the optimizer function of the Design expert software. Considering the cost for HMFSs production and acceleration of denaturation rate of enzyme under high temperature, suitable substrate molar ratio and lower temperature were preferred in selection of reaction conditions. Therefore, the predicted optimal conditions were chosen as: reaction time, 3.4 h; temperature, 57 °C; substrate molar ratio,

Table 5. FA Composition and Position Distribution of HMFSs Produced under Optimum Conditions

FA (mol %)	total	sn-2	sn-1,3[a]
C12:0	3.1	0.4	4.5
C14:0	4.3	0.7	6.1
C16:0	29.7	62.8	13.1
C18:0	7.2	6.6	7.5
C18:1n-9	34.4	22.7	40.2
C18:2n-6	18.2	6.1	24.3
C18:3n-3	2.9	0.4	4.1

[a] Indicates FA composition at the sn-1,3 positions, calculated as (3 × total − sn-2)/2.

14.6 mol/mol; and enzyme load, 10.7 wt %, and the predicted values for content of PA and sn-2 PA were 29.8 and 62.1%, respectively, which were within the expected values.

Model Verification. Reactions were carried out at the predicted optimal conditions to validate the models, and the results of FA composition and position distribution of the final product are presented in Table 5. The experimental values for the content of PA (29.7%) and sn-2 PA (62.8%) agreed well with the predicted values (29.8 and 62.1%, respectively), which indicated the validation of both models, and the deviations between the predicted and the experimental results were probably due to some errors in the control of reaction conditions. In addition, other FAs in the product, including lauric acid (3.1%), myristic acid (4.3%), stearic acid (7.2%), oleic acid (34.4%), linoleic acid (18.2%), and linolenic acid (2.9%) were all within the range of the corresponding FAs of HMF, and the ratio of linoleic acid to linolenic acid was 6.3, similar to that of HMF.[4]

Evaluation of HMFSs. The degree of similarity of the product obtained under optimum conditions to HMF was evaluated by the cited model.[27] The scores for similarity of total FAs (G_1) and sn-2 FAs (G_2) were 44.2 and 39.5, respectively, and the total score (G) for the product was 83.7, which indicated high degree of similarity of the product. Therefore, on the basis of above-mentioned results, the product can be used as a substitute for HMF in infant formula.

Reusability of Lipozyme RM IM. One of the important advantages of immobilized enzymes as catalysts is their reusability. However, the catalytic activity irreversibly decreases as recycling times increase, which is probably due to the intensive mechanical agitation and high temperature destroying the structure of the enzymes. The kinetic properties of enzymes determine the final properties of the product; that is, the distortion of the enzymes may affect not only the initial rate but also the final distribution of FAs. Therefore, to ensure the quality of the product, the stability and reusability of the enzyme should be investigated during the reactions. In this experiment, the initial activity of the enzyme, defined as the content of PA of the product after 30 min of reaction, and the contents of PA in the final produced SLs were selected to evaluate the stability and reusability of lipozyme RM IM. Figure 6 shows the variations of the initial activity of the enzyme and content of PA of the final product at different recycling runs. The immobilized lipase could be used at least 17 cycles without significant loss of initial activity, and the content of PA was less than 32% after three more recycling runs. Good behavior of lipozyme RM IM might benefit from the immobilization, which rigidifies the enzyme structure[21] and the relatively mild reaction conditions. However, 17 cycles

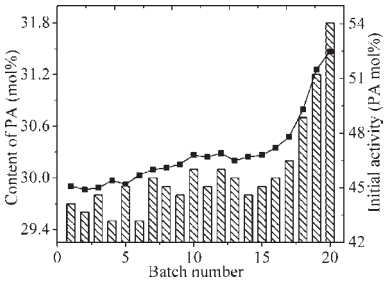

Figure 6. Reusability of lipozyme RM IM in the acidolysis of interesterified palm stearin with mixed FAs. Reaction conditions: reaction time, 3.4 h; temperature, 57 °C; substrate molar ratio, 14.6 mol/mol; and enzyme load, 10.7 wt %. Content of PA, hatched bars; and initial activity, black bars.

are not enough to make the process economically viable. Some new methods for immobilization (e.g., multipoint covalent attachment) or new bioreactors (e.g., packed bed reactor), which can further improve the stability of the enzyme during reaction, therefore, will be developed in the future study.

In conclusion, the preparation of HMFSs by acidolysis of palm stearin with mixed FAs catalyzed by lipozyme RM IM was successfully modeled and optimized by RSM, and a high degree of similarity of the HMFSs to HMF was also proved by the cited model. The optimized conditions were then given as follows: reaction time, 3.4 h; temperature, 57 °C; substrate molar ratio, 14.6 mol/mol; and enzyme load, 10.7 wt %. Under these conditions, the contents of PA and sn-2 PA were 29.7 and 62.8%, respectively, and the score for degree of similarity of the product was 83.7. Lipozyme RM IM in the present work can be used at least 17 times. These results suggest that it is practical to prepare HMFSs from palm stearin by enzymatic acidolysis.

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