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Enzymatically Catalyzed Synthesis of Anti-blooming Agent 1,3-Dibehenoyl-2-oleoyl Glycerol in a Solvent-Free System: Optimization by Response Surface Methodology

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Supporting Information

ABSTRACT: Products rich in 1,3-dibehenoyl-2-oleoyl glycerol (BOB) triglyceride (TAG) were produced by enzymatic interesterification of high oleic acid sunflower oil (HOSO) and behenic acid methyl ester (BME) by 1,3-regiospecific lipase Lipozyme RM IM in a solvent-free system. The impact factors of enzyme load, substrate molar ratio of BME to HOSO (BME/HOSO), reaction time, reaction temperature, and pre-equilibration water activity of the enzyme on BOB content and BME conversions were investigated by single-factor experiments and then optimized using the response surface methodology (RSM). The optimum conditions were as follows: reaction temperature, 72 °C; reaction time, 7.99 h; substrate molar ratio, 2.5:1; enzyme load, 10%; and pre-equilibration water activities of the enzyme, 0.28. The results from the experiments conducted according to the predicted optimal conditions were as follows: the content of BOB was 32.76%, and the conversion of BME was 65.16%. The experimental values agreed with the predicted values, which verified the sufficiency of the quadratic regression models. After purification under the optimal short-range molecular distillation and two-step solvent fractionation, the content of BOB in the target product can reach 77.14%, indicating the great potential for industrial production of the anti-blooming agent.

KEYWORDS: *Anti-blooming agent, 1,3-dibehenoyl-2-oleoyl glycerol, enzymatic interesterification, response surface methodology*

INTRODUCTION

Fat bloom in chocolates has been researched for many decades. It can lead to the decline in product quality, which is a worldwide problem in the confection industry. A bloomed chocolate is characterized by the loss of the initial gloss of the surface, giving rise to a more or less white aspect. The bloom in chocolate depends upon many factors. There are many corresponding strategies that have been proposed for attenuating the formation of the bloom in chocolate. Many researchers described the effects of emulsifiers,[1−3] some natural substances,[4] and milk fat or its fractions[5] on the bloom in chocolate. Some special triglycerides (TAGs), such as 1,3-distearoyl-2-oleoyl glycerol (SOS), 1,3-oleoyl-2-stearoyl glycerol (OSO), and 1,3-dibehenoyl-2-oleoyl glycerol (BOB), have also been acting as bloom inhibitors.[6,7]

BOB is a mixed-acid TAG, in which R_1 = R_3 = behenic acid and R_2 = oleic acid. Wang et al.[8] described the six forms of BOB, α, β', γ, pseudo-β', β_2, and β_1. Among them, β_2 of BOB has a high melting point of 52 °C. The use of BOB as the seed crystal material in the cocoa butter crystallization has seen excellent advantages. Hachiya et al. found that the seeding of β_2 of BOB caused better demolding β_V form crystallization of cocoa butter and the most preferable fat-bloom stability. When the seeding of β_2 of BOB dosages based on the weight of total substances increased to 5%, the fat bloom after thermo cycle tests between 38 and 20 °C had been completely prevented.[6,7] Momura et al.[9] invented a fat-blooming inhibitor, which was synthesized by behenic acid acidolysis of olive oil with a lipase originating from *Rhizopus delemar* in the *n*-hexane system. The inhibitor contains 52% behenic acid, and thermo cycle tests between 30 and 20 °C confirmed the action of this inhibitor on the fat bloom. Padley et al.[10] carried out enzymatic interesterification of high erucic rapeseed oil with a mixture of fatty acids, which contain behenic acid (obtained by hydrolyzing a hardened rapeseed oil) in the *n*-hexane media, after a refining process, 19.5% dibehenoyl-containing TAGs, and 44.7% monobehenoyl-containing TAGs presented in the interesterified product. It proved that the presence of 5−7% of BOB in chocolate showed good anti-blooming properties. Because natural oils and fats nearly do not contain BOB, a serious problem, which BOB is extremely expensive, still remains. Yoon et al.[11,12] synthesized BOB through the interesterification reaction of triolein by behenic acid or ethyl behenate in supercritical carbon dioxide ($SCCO_2$), and ethyl behenate was found to give the higher production rate of the final BOB production. Kojima et al.[13] investigated the antiobesity activities of dietary 1-behenoyl-2,3-dioleoyl glycerol (BOO) in rats. Their research involved using sunflower oil and behenic acid ethyl ester to synthesis a BOO-rich experimental oil sample. A kind of TAG forming in the experimental oil was BOB, but its content only reached 6.5%.

In summary of the associated reports on producing BOB, most of the studies were completed in the laboratory and hardly applied in the industry. Some researchers used hydrogenated feedstock to obtain BOB; this method was

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proven to be complicated and had trouble with fractionation. There were some related reports about synthesizing BOB in solvent media but nearly no report about synthesizing a BOB-rich experimental oil in a solvent-free system. The solvents not only increase the industrial cost but also have an adverse effect on health.[14] In our study, products rich in BOB were produced via enzymatic interesterification of high oleic acid sunflower oil (HOSO) and behenic acid methyl ester (BME) by 1,3-regiospecific lipase in a solvent-free system. In comparison to behenic acid, BME has a lower melting point, which makes it possible to reduce the reaction temperature. Lipozyme RM IM immobilized from *Rhizomucor miehei* was selected as the catalyst. It was proven that Lipozyme RM IM is 1,3-regiospecific and has higher activity compared to Lipozyme TL IM and Novo 435 in the reaction of triolein with behenic acid.[15]

Response surface methodology (RSM) can evaluate the effects of multiple parameters on response variables alone or in combination. It is the most commonly used tool, which can help optimize the process with a reduced number of experiments.[16,17] The research aim of this study is to optimize the reaction conditions (reaction time, temperature, substrate molar ratio, enzyme load, and pre-equilibration water activities of the enzyme) by the single factor and RSM for maximum yield of BOB contents and the conversion of BME. Our investigations found that the reaction mainly produced BOB and BOO. To obtain higher BOB, short-range molecular distillation and fractional extraction with acetone were used to concentrate BOB. Therefore, the present study may be able to give a commercially viable method for industrial production of the anti-blooming agent.

MATERIALS AND METHODS

Materials. HOSO [containing 5.15% stearic acid ($C_{18:0}$), 80.92% oleic acid ($C_{18:1}$), and 9.64% linoleic acid ($C_{18:2}$)] was produced by Joyful Organic Co., Ltd. (Beijing, China). Lipozyme RM IM (from *R. miehei*), a commercially immobilized 1,3-specific lipase, was obtained from Novozymes A/S (Bagsvaerd, Denmark). BME (purity ≥ 85%) was purchased from Sichuan Sipo Chemcial Co., Ltd. (Sichuan, China). BOB standard (99% purity) and 1,2-dibehenoyl-3-oleoyl glycerol (BBO) standard (99% purity) were purchased from Larodan Co. (Sweden). Supelco 37 Component FAME Mixture was purchased from Sigma-Aldrich China (Shanghai, China). All other analytical- or chromatographic-grade reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Interesterification. The interesterification reactions were carried out in glass reactors with a jacket. The vials were connected to a super constant temperature water bath and used a multiple magnetic stirrer to mix the HOSO and BME (vacuum drying 6 h under the condition of 55 °C and 0.1 MP) while keeping them at a certain temperature for 5 min and injecting nitrogen, and then the Lipozyme RM IM was added. The effects of reaction times ranged from 4 to 12 h; substrate molar ratios (BME/HOSO) ranged from 1.5:1 to 4:1; enzyme dosages ranged from 4 to 14% (on the basis of total substrates in weight); temperatures ranged from 55 to 85 °C; and pre-equilibration water activities of the enzyme ranged from 0.28 to 0.76 on the incorporations studied. Initial water activities (a_w) of the enzyme were performed following the procedures of Lue et al.,[18] which were adjusted prior to activity measurement by placing the Lipozyme RM IM in sealed vessels containing a wide range of saturated aqueous salt solutions, including $MgCl_2$ (a_w = 0.33), $LiNO_3$ (a_w = 0.48), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), and KCl (a_w = 0.84). Water activity equilibration was conducted for 7 days at room temperature. The practical water activity values of the enzyme under the above different conditions were measured by a water activity meter (GBX FAst/lab, Romans sur Isère Cedex, France), which were 0.28 ($MgCl_2$), 0.40 ($LiNO_3$), 0.47 [$Mg(NO_3)_2$], 0.65 (NaCl), and 0.76 (KCl). All reactions and measurements were duplicated.

Fatty Acid Composition. Fatty acid methyl esters (FAMEs) were analyzed by GC-14B gas chromatography (GC) equipped with a flame ionization detector (Shimadzu, Tokyo, Japan). A fused-silica capillary column (CP-Sil88, 100 m × 0.25 mm × 0.2 μm) was used to analyze the samples, which were pretreatment according to the American Oil Chemists' Society (AOCS) Official Method Ce 2-66.[19] The temperature of the injection port and detector were all maintained at 250 °C. The column temperature was held at 80 °C for 3 min, then programmed at 15 °C/min to 215 °C, and held for 15 min. The fatty acid species were identified with standard FAMEs.

TAG Composition. Reversed-phase high-performance liquid chromatography (RP-HPLC) with a Waters 1525 binary pump (Milford, MA) was used to separate the TAGs of the reaction product. Samples were dissolved in chloroform at a concentration of 3.0 mg/mL and injected into a Nova-pak RP-C18 column (150 × 4.6 mm, particle size of 4 μm, Waters, Milford, MA), and the injection volume was 8 μL. The column temperature was kept at 35 °C. We use acetonitrile as mobile phase A and *n*-hexane/isopropanol (1:1, v/v) as mobile phase B at a flow rate of 1.0 mL/min. The gradient was as follows: 0 min, 60% acetonitrile; 25 min, 40% acetonitrile; 30 min, 40% acetonitrile; 31 min, 60% acetonitrile; and 35 min, 60% acetonitrile. An Alltech 3300 (Grace Davision Discovery Sciences, Deerfield, IL) evaporative light scattering detector (ELSD) was used. The ELSD was set to 55 °C at a gain of 1, and the flow rate of high-purity nitrogen nebulizer gas was set to 1.8 mL/min.

BOB and BBO both have the same chain length and degree of unsaturation of fatty acid; therefore, the RP-HPLC cannot separate them, but preparative HPLC and silver-ion HPLC can be used as the complements to RP-HPLC. A Sunfire C18 column (150 × 19 mm) was used in the preparative HPLC to prepare the BOB/BBO solution. The constant column flow rate was 8 mL/min, and the injection volume was 250 mL. The other conditions were the same as the method of RP-HPLC above. The silver-ion HPLC was used to separate and for quantification of BOB and BBO positional isomers according to the methods reported by Chen et al.[20] and Dugo et al.[21] A silver-ion chromatographic column was ChromSpher Lipids (250 × 4.6 mm, 5 μm, Varian, Palo Alto, CA), and the column temperature was kept at 30 °C. The mobile phase was *n*-hexane/ethyl acetate/toluene (8:1:1, v/v/v). The other conditions were also the same as the method of RP-HPLC above. All TAG contents were given in percentage area. TAGs separated by RP-HPLC were identified by high-performance liquid chromatography/atmospheric pressure chemical ionization−mass spectrometry (HPLC/APCI−MS). Qualitative and quantitative determinations of individual TAGs in samples were performed following the procedures previously reported by our team.[22] The TAG species separated by the silver-ion HPLC was determined by the retention time of the TAG standard solution.

Purification of the Interesterification Product. To obtain more purity BOB, short-range molecular distillation and fractional extraction with acetone were used to refine the reaction product. A molecular distillation equipment (KDL1, UIC, Germany) was used to remove the fatty acid methyl esters. Two vamp pumps and a diffusion pump are composed of the vacuum system. To provide the evaporator heat, a super constant temperature oil bath was used to give the circulating heated oil. The parameters of the process were as follows: distillation temperature, 170 °C; rotate speed of the wiped film, 120 rpm; feed speed, 2 mL/min; vacuum degree, 9 × 10^{-3} MP; preheating temperature, 65 °C; and condensate temperature, 55 °C. The heavy phase was the target product. To reduce the level of tribehenin (BBB) and BOO, a two-step solvent fractionation method was adopted to treat the heavy phase. A jacketed reaction system (Reactor-Ready Lab Reactor) connected to a LADUA ECO (RE630, LADUA, Germany) was used to control the crystallization process. The heavy phase was fractionated by acetone, with oil/solvent ratios of 1:4 (w/v). The first step of crystallization was held at 58 °C for 20 min, reduced to 40 °C in 18 h, and finally kept at 40 °C for 6 h. Then, the liquid fraction was conducted to the second step of crystallization, which was held at 58

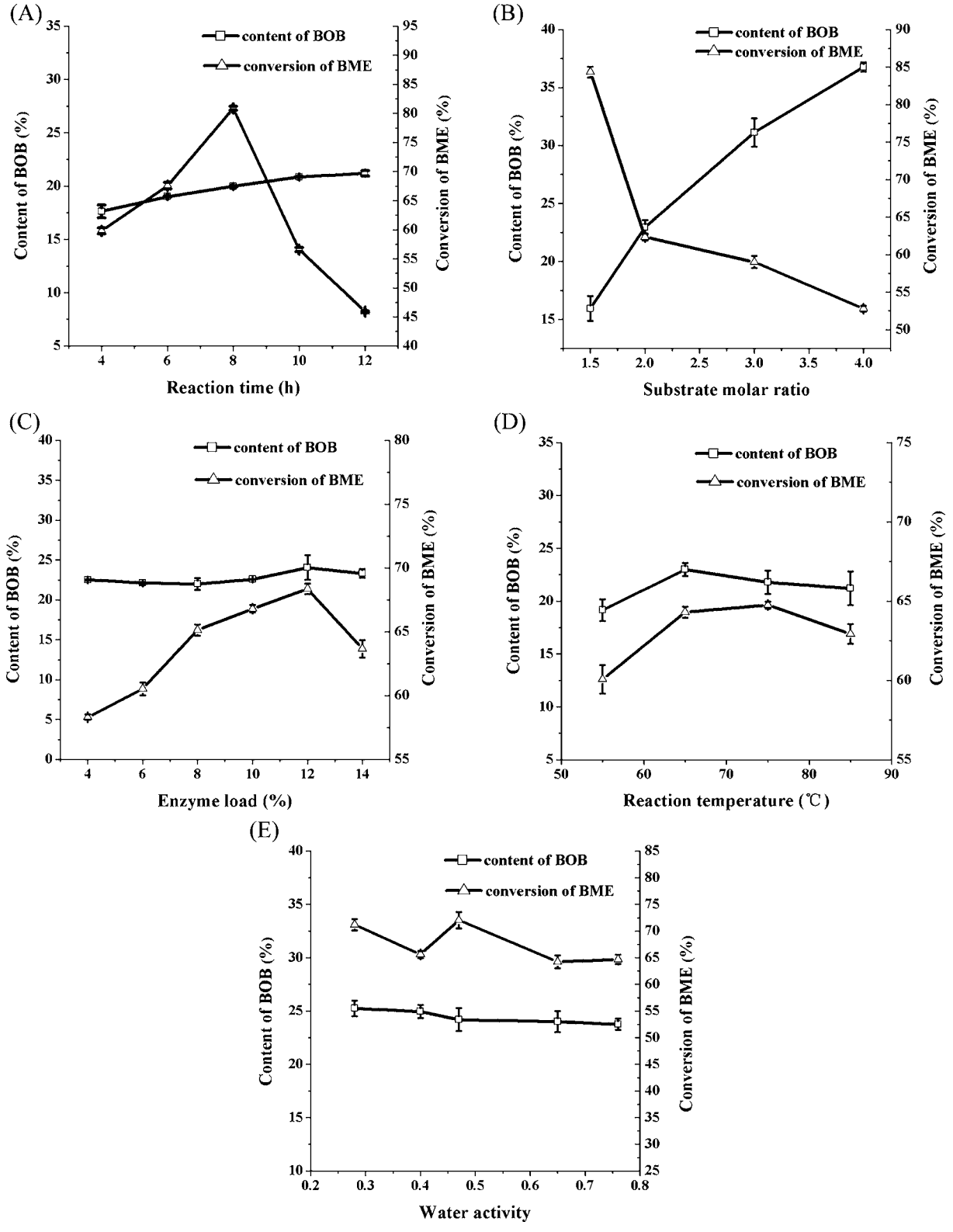

Figure 1. Effects of the reaction time, substrate molar ratio (BME/HOSO), enzyme load (on the basis of total substrates in weight), reaction temperature, and pre-equilibration water activities of the enzyme on the conversion of BME (△) and the content of BOB (□): (A) reaction temperature, 65 °C; substrate molar ratio (BME/HOSO), 2:1; enzyme load (on the basis of total substrates in weight), 10%; and pre-equilibration water activities of the enzyme, 0.28; (B) reaction temperature, 65 °C; reaction time, 8 h; enzyme load (on the basis of total substrates in weight), 10%; and pre-equilibration water activities of the enzyme, 0.28; (C) reaction temperature, 65 °C; reaction time, 8 h; substrate molar ratio (BME/HOSO), 2:1; and pre-equilibration water activities of the enzyme, 0.28; (D) reaction time, 8 h; substrate molar ratio (BME/HOSO), 2:1; enzyme load (on the basis of total substrates in weight), 10%; and pre-equilibration water activities of the enzyme, 0.28; and (E) reaction temperature, 65 °C; reaction time, 8 h; substrate molar ratio (BME/HOSO), 2:1; and enzyme load (on the basis of total substrates in weight), 10%.

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Table 1. Regression Analysis of Variance for the Response Surface Quadratic Model (ANOVA) Pertaining to the Predicted Content of BOB

source	degree of freedom	sum of squares	mean square	F value	prob > F^a
model	9	413.20	45.91	60.42	<0.0001
X_1	1	28.01	28.01	36.86	0.0005
X_2	1	68.21	68.21	89.76	<0.0001
X_3	1	273.20	273.20	359.52	<0.0001
X_1X_2	1	6.40	6.40	8.42	0.0229
X_1X_3	1	0.26	0.26	0.34	0.5805
X_2X_3	1	5.06	5.06	6.66	0.0364
X_1X_1	1	1.992×10^{-3}	1.992×10^{-3}	2.621×10^{-3}	0.9606
X_2X_2	1	31.94	31.94	42.03	0.0003
X_3X_3	1	0.069	0.069	0.091	0.7715
residual	7	5.32	0.76		
lack of fit	3	0.36	0.12	0.096	0.9585[b]
pure error	4	4.96	1.24		
correlation total	16	418.52			
CV = 1.99%	adjusted R^2 = 0.9979				

[a] $p < 0.05$ indicates statistical significance. [b] $p > 0.05$ indicates that the lack of fit is not significant, which demonstrates that the specified model is adequate.

Table 2. Regression Analysis of Variance for Response Surface Quadratic Model (ANOVA) after Backward Elimination Pertaining to the Predicted Conversion of BME

source	degree of freedom	sum of squares	mean square	F value	prob > F^a
model	9	1089.97	121.11	148.37	<0.0001
X_1	1	61.26	61.26	75.05	<0.0001
X_2	1	22.38	22.38	27.42	0.0012
X_3	1	755.28	755.28	925.30	<0.0001
X_1X_2	1	7.39	7.39	9.05	0.0197
X_1X_3	1	30.71	30.71	37.62	0.0005
X_2X_3	1	9.89	9.89	12.12	0.0103
X_1X_1	1	67.22	67.22	82.36	<0.0001
X_2X_2	1	0.65	0.65	0.80	0.4009
X_3X_3	1	144.62	144.62	177.17	<0.0001
residual	7	5.71	0.82		
lack of fit	3	1.64	0.55	0.54	0.6820[b]
pure error	4	4.07	1.02		
correlation total	16	1095.69			
CV = 1.32%	adjusted R^2 = 0.9881				

[a] $p < 0.05$ indicates statistical significance. [b] $p > 0.05$ indicates that the lack of fit is not significant, which demonstrates that the specified model is adequate.

°C for 20 min, reduced to 25 °C in 18 h, and finally kept at 25 °C for 6 h. The solid fraction was the finally purified product.

Statistical Analysis. The data were analyzed by the response surface procedure (Design Expert, State-Ease, Inc., Statistics Made Easy, Minneapolis, MN; version 5.0.7.1997). Box–Behnken response design for three factors and three levels was used to obtain the effects of the substrate molar ratio, temperature, and reaction time on the interesterification to optimize the processing conditions. High responses of BME conversion and BOB content were expected.

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

The experimental design included 17 tests of three variables at three levels (−1, 0, and +1). X_i and X_j are the coded independent variables, and β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for the intercept, linear, quadratic, and cross-product terms, respectively.

RESULTS AND DISCUSSION

Selection of Independent Variables and Their Levels. The effects of five independent variables on BME conversion and BOB content in the reaction product were shown in Figure 1. Figure 1A showed that the content of BOB was increased with the increase of the reaction time. When the reaction time increased from 4 to 8 h, the conversion of BME increased to 80.89%. The content of BOB increased from 15.94 to 36.76% when the substrate molar ratio increased from 1.5:1 to 4:1, respectively. It was shown that the value for the content of BOB quickly increased among the experimental variables tested. The conversion of BME was decreased quickly with the increase of the substrate molar ratio (Figure 1B). The plot for the content of BOB and conversion of BME versus enzyme load was given in Figure 1C. The highest responses (24.05 and 68.36%, respectively) were obtained with the 12% enzyme dosage. This result can be explained by the fact that the higher enzyme load caused the problem of biocatalyst agglomeration and the diffusion of substrates. However, the enzyme dosage of 10% was chosen in the present study to reduce the production costs. The conversion of BME and the content of BOB all showed increasing–decreasing patterns when the reaction temperature

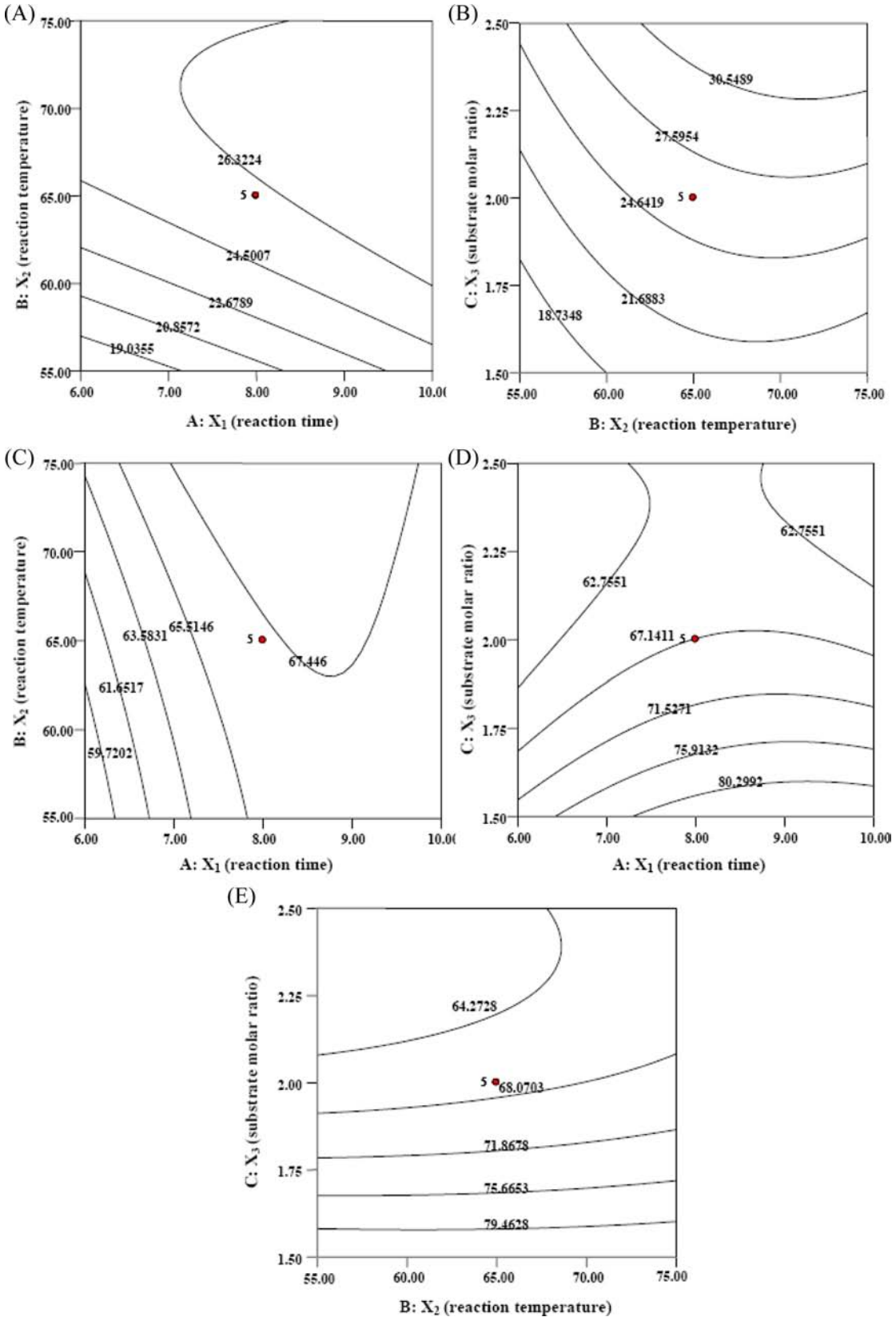

Figure 2. Contour plots of the (A and B) content of BOB and (C–E) conversion of BME under fixed enzyme load (on the basis of total substrates in weight) of 10% and pre-equilibration water activities of the enzyme of 0.28: (A) substrate molar ratio (BME/HOSO), 2:1; (B) reaction time, 8 h; (C) substrate molar ratio (BME/HOSO), 2:1; (D) reaction temperature, 65 °C; and (E) reaction time, 8 h.

increased (Figure 1D). Because of the high melting point of BME, a high temperature is essential to reduce the viscosity of the system. Effects of pre-equilibration water activities of the enzyme on the content of BOB and the conversion of BME

were shown in Figure 1E. With other variables fixed, the content of BOB was decreased slowly as the water activity increased. The conversion of BME showed no clear trend.

In this study, effects of the enzyme load and pre-equilibration water activities of the enzyme on the changes of BME conversion or BOB content were slight. Overall, reaction time, reaction temperature, and substrate molar ratio had more influence on BOB content and BME conversion. With setting of the enzyme load and pre-equilibration water activities of the enzyme at 10% and 0.28, respectively, the lower, middle, and upper levels of the three independent variables were chosen in Table S1 of the Supporting Information. Y_1 and Y_2 are the predicted value for the content of BOB (%) and the conversion of BME (%), respectively. X_1, X_2, and X_3 are the coded variables, as described in Table S1 of the Supporting Information.

Analysis of the Models. The responses of the BOB content and BME conversion obtained from all of the experiments were shown in Table S2 of the Supporting Information according to RSM design. The analysis of variance (ANOVA) of the second-order models for two response variables was listed in Tables 1 and 2. Two quadratic regression models were given as follows:

$$Y_1 = 26.05 + 1.87X_1 + 2.92X_2 + 5.84X_3 - 1.26X_1X_2 - 0.25X_1X_3 + 1.13X_2X_3 - 0.022X_1^2 - 2.75X_2^2 + 0.13X_3^2 \quad (2)$$

$$Y_2 = 67.19 + 2.77X_1 + 1.67X_2 - 9.72X_3 - 1.36X_1X_2 - 2.77X_1X_3 + 1.57X_2X_3 - 4.00X_1^2 + 0.39X_2^2 + 5.86X_3^2 \quad (3)$$

F and p values determined the significance of every coefficient. According to the ANOVA date listed in Tables 1 and 2, in this study, most terms were significantly below 0.05, except for the test variables, which were insignificant model terms with p values greater than 0.05. This indicated that the real relationship between the responses and the significant variables explained by the models were prominent and acceptable. The insignificant terms were not eliminated from the model to retain the hierarchy of the model. The coefficients of determination (R^2) of the models for the content of BOB and conversion of BME were 0.9873 and 0.9948, respectively. The adjusted coefficients of determination 0.9979 and 0.9881 indicated that the response surface model can explain more than 99.79 and 98.81% of the variation for the response variables studied, respectively. Figure S1 of the Supporting Information indicated that the correlations between the predicted and observed BOB and BME responses were satisfactory. The lack of fits of the two models (content of BOB, 0.9585; conversion of BME, 0.6820) were both insignificant, which indicated that the models were perfectly suitable.

As the coefficient of the linear terms of the two models shown in Tables 1 and 2, the most important factor that affects the content of BOB and conversion of BME was the substrate molar ratios while also showing that the order of reaction variables affecting the BOB content were as follows: substrate molar ratio > reaction temperature > reaction time. The order of reaction variables affecting BME conversion were as follows: substrate molar ratio > reaction time > reaction temperature. The contour plots of the quadratic model (Figure 2) showed the interactive effects of the reaction time, substrate molar ratio, and reaction temperature. Generally, an increment in the reaction temperature can increase the conversion of BME. The BME conversion showed increasing−decreasing patterns when the reaction time increased. The lowest of the substrate molar ratio would gain the maximum BME conversion. The BOB content also showed increasing−decreasing patterns when the reaction temperature increased. The higher the other two variables, the higher the BOB content.

Effect of the Temperature. The reaction temperature is a key factor in the production of structured lipids. The affinities between enzyme and substrate, enzyme stability, and content of byproduct were affected by the reaction temperature.[23] In the present study, it is said that Lipozyme RM IM was more active at 40 °C.[15] However, the contour plots (panels A and B of Figure 2) showed that the yield of BOB had a maximum value with the temperature within the range of 65−73 °C, while the reaction temperature continues rising, the content of BOB had little decrease. In conclusion, in our study, Lipozyme RM IM was more active at higher temperatures when the temperature was below 73 °C. The conversion of BME increased with the increase of the temperature. There are some possible reasons to explain this result. BME has a high melting point, because a higher temperature could increase the substrate dissolution and decrease the solution viscosity;[24] therefore, a higher BOB content can be obtained when the temperature rises. A higher temperature increases the reaction rate because it reduces the viscosity of the lipid mixture and increases the substrate and product transfer on the surface or inside the enzyme particles, and it has been reported that a higher temperature favors higher yields for endothermic reactions because of the shift of thermodynamic equilibrium. However, when the temperature further increased to 75 °C, the enzyme stability and its half-life were greatly reduced and the content of byproduct was increased.[23] Therefore, a higher temperature was not wanted.

Effect of the Reaction Time. The reaction time was set at 6, 8, and 10 h to investigate the influence of the reaction time on the content of BOB and conversion of BME. The contour plots (panels A, C, and D of Figure 2) showed that the amount of BOB increased with the increasing reaction time. When the other two reaction conditions are kept at a constant level, there is little decrease in the conversion of BME when the reaction time is very close to 10 h. It was reported that increased acyl migration was caused by a longer reaction time. Increased acyl migration causes the reduced yield of target production by increasing the TAGs composed of other fatty acids at the Sn-2 position.[25]

Effect of the Substrate Molar Ratio. The substrate molar ratio exerts an important effect on enzymatic interesterification. In the present study, it was reported that a higher molar ratio of acyl donors yielded higher acyl incorporation.[26] The higher substrate molar ratio can raise the reaction equilibrium and increase the ratio of the collision between substrates and catalyst.[27] The molar ratio of BME to HOSO was varied from 1.5:1 to 2.5:1. Three contour plots for the effect of the substrate molar ratio to the content of BOB and conversion of BME were shown in panels B, D, and E of Figure 2. As the substrate molar ratio increased, the content of BOB increased. As discussed in the analysis of the models before, the main reason behind this increase is the effect of the substrate molar ratio. However, the conversion of BME decreased with the substrate molar ratio increasing. Peng et al.[28] reported about the effect of the

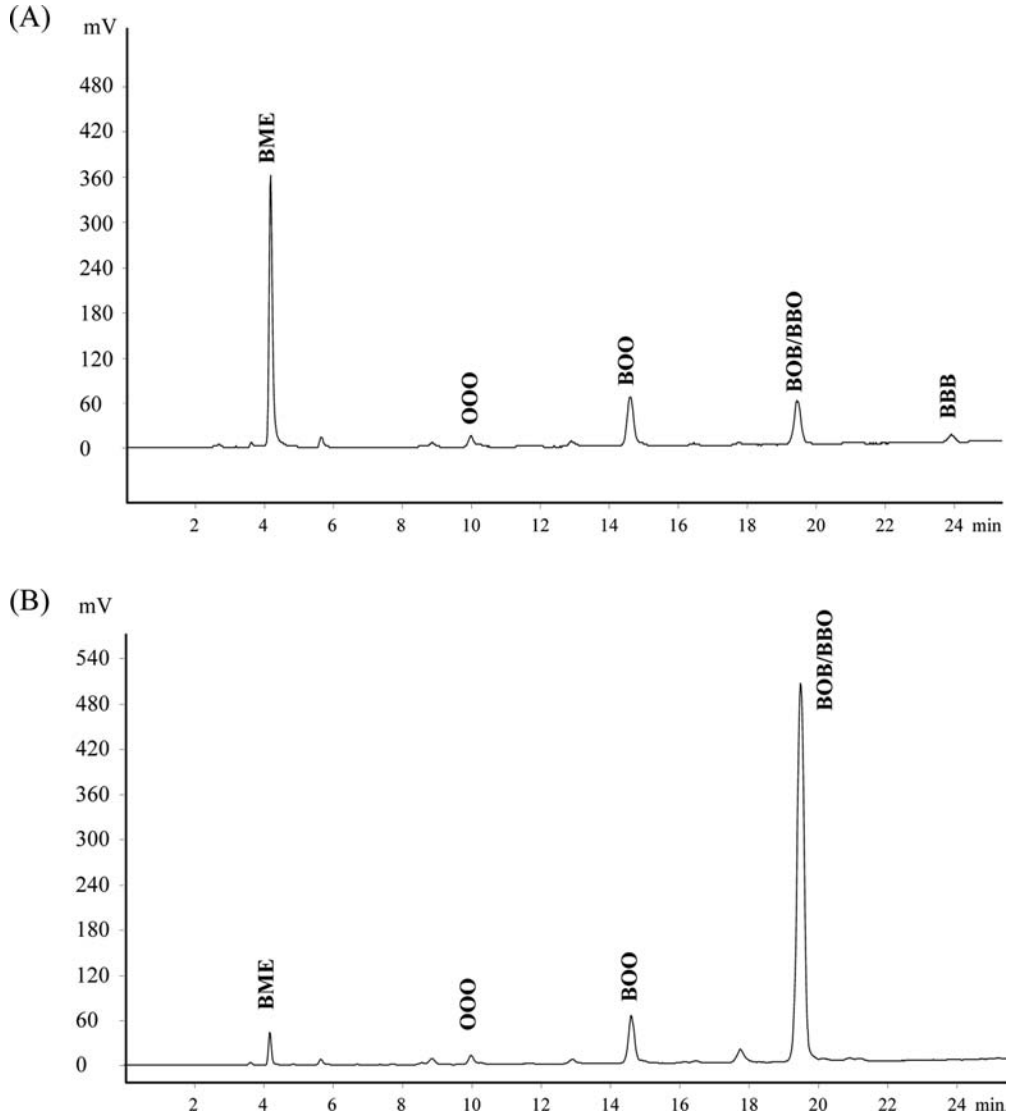

Figure 3. HPLC−ELSD chromatograms of the purified interesterification product: (A) before purification and (B) after purification.

substrate molar ratio on the reaction. They also found that a higher substrate molar ratio was beneficial to acyl incorporation from the view of reaction equilibrium. In conclusion, a higher substrate molar ratio caused a higher BOB content, resulting in a decreased conversion of BME. A conversion of BME greater than 65% was wanted to improve the utilization of BME.

Optimization of the Reaction and Model Verification. The conversion of BME and the content of BOB were used for maximization values. The optimum conditions yielding 33.49% of BOB and 65.82% conversion of BME were obtained as follows: a reaction temperature of 72 °C, a reaction time of 7.99 h, and a substrate molar ratio of 2.5:1. The results from the experiments conducted according to the predicted optimal conditions were as follows: the content of BOB was 32.76%, and the conversion of BME was 65.16%. The experimental values agreed with the predicted values, which verified the sufficiency of the quadratic regression models.

Purification of the Interesterification Product. The interesterification product contains the target BOB (32.76%), some other TAGs, such as BOO (34.35%), OOO (8.74%), BBB (4.60%), and BBO (0.45%), a little monoglycerides (0.90%) and diglycerides (2.72%), unreacted substrate, and some FAMEs. The amounts of monoglycerides and diglycerides in the reaction product were low. They can make food more homogeneous and are usually used as an emulsifier in the food industry. Therefore, it is unnecessary to take them off. After molecular distillation and two-step solvent fractionation, most BME, BOO, and BBB at nearly 4.2, 14.6, and 24.0 min, respectively, had been taken off, which was obvious by comparing panels A and B of Figure 3. The major fatty acids of the reaction materials and the purified interesterification products were $C_{18:1}$ (36.78 and 31.01%, respectively) and behenic acid ($C_{22:0}$, 53.97 and 63.24%, respectively). After purification, the content of BOB in the product can reach 77.14%, also containing BOO (9.17%), OOO (1.53%), BBB (2.85%), BBO (1.02%), and diglycerides (4.70%).

In the present study, the BOB-rich target product, a kind of anti-blooming agent for chocolate, composed of behenic and oleic acids, was successfully synthesized with Lipozyme RM IM. On the basis of the single factor, RSM was used to model and optimize the process. The optimal conditions were as follows: reaction temperature, 72 °C; reaction time, 7.99 h; substrate molar ratio, 2.5:1; enzyme load, 10%; and pre-equilibration water activities of the enzyme, 0.28. Under these optimal

conditions, the conversion of BME and content of BOB actually achieved were 65.16 and 32.76%, respectively. After purification, under the optimal short-range molecular distillation and two-step solvent fractionation, the content of BOB in the target product can reach 77.14%, which showed the great potential for industrial production of the anti-blooming agent.

■ ASSOCIATED CONTENT

Supporting Information

Variables (factors) used for central composite design (Table S1), experimental data for the three-factor, three-level surface analysis (Table S2), and relationship between the observed and predicted (A) BOB content responses and (B) BME conversion responses (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

BOB, 1,3-dibehenoyl-2-oleoyl glycerol; TAG, triacylglycerol; HOSO, high oleic acid sunflower oil; BME, behenic acid methyl ester; RSM, response surface methodology; SOS, 1,3-distearoyl-2-oleoyl glycerol; OSO, 1,3-oleoyl-2-stearoyl glycerol; $SCCO_2$, supercritical carbon dioxide; BOO, 1-behenoyl-2,3-dioleoyl glycerol; BBO, 1,2-dibehenoyl-3-oleoyl glycerol; a_w, water activity; FAME, fatty acid methyl ester; GC, gas chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; ELSD, evaporative light scattering detector; HPLC/APCI−MS, high-performance liquid chromatography/atmospheric pressure chemical ionization−mass spectrometry; BBB, tribehenin; ANOVA, analysis of variance

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