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Synthesis of 1,3-distearoyl-2-oleoylglycerol by enzymatic acidolysis in a solvent-free system

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ABSTRACT

1,3-Distearoyl-2-oleoylglycerol (SOS) is widely used as a cocoa butter improver and an anti-blooming agent in the chocolate industry. In this study, an effective process was developed to prepare SOS by enzymatic acidolysis. Under optimal reaction conditions (substrate molar ratio of 12, NS40086 loading of 10%, a solvent-free system, 75 °C for 4 h), a SOS yield of 70.2% was obtained. Subsequently, two-step purification was applied to purify the crude SOS product. Free fatty acids were completely removed after molecular distillation. After acetone fractionation, the purity of SOS reached 92.2% with an 85.1% recovery. Comparison of various physicochemical properties of purified SOS and a commercial cocoa butter improver showed their properties to be similar. This study provides an effective and sustainable process for the synthesis of SOS product, which is expected to be used as a high-quality cocoa butter improver or an anti-blooming agent in the chocolate industry.

1. Introduction

Cocoa butter is one of the most expensive and valuable raw materials used in the production of chocolate in the confectionery industry due to its unique physicochemical properties. The key ingredients in chocolate are cocoa solids, cocoa butter, sugar and lecithin as an emulsifier. However, the wide diversity of products available on the market is the result of incorporating other compounds in the formulations. Cocoa butter is mainly composed of three triacylglycerols (TAGs) with oleic acid in the *sn*-2 position: 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1-palmitoyl-3-stearoyl-2-oleoylglycerol (POS) and 1,3-distearoyl-2-oleoylglycerol (SOS) (Çiftçi, Fadıloglu, & Gögüs, 2009). The unique TAG composition of cocoa butter provides chocolate with snap, gloss and a smooth mouth feel. Although cocoa butter is an ideal fat for use in chocolate, its use is limited in tropical countries because it softens with heat and tempering is difficult (Jahurul et al., 2014). In addition, when chocolate products are improperly stored and insufficiently tempered, fat bloom may occur (Naosuke, Miho, Shuichi, & Tsugio, 1999; Peschar et al., 2004). Fat bloom is very difficult to prevent even if the products are subjected to an excellent tempering process (Naosuke et al., 1999), and it remains a major concern for many chocolate manufacturers as it leads to the formation of an undesirable white, dusty surface appearance.

To avoid fat bloom, many strategies have been suggested to inhibit its formation in chocolate by mixing cocoa butter with its equivalents or improvers. For example, the addition of a high-melting milk-fat fraction to cocoa butter can inhibit fat bloom (Bricknella & Hartel, 1998). Additionally, some special TAGs, such as SOS and 1,3-dibehenoyl-2-oleoylglycerol (BOB), have been used as agents to inhibit fat bloom (Maheshwari & Reddy, 2005; Meng et al., 2013). SOS not only exhibits an anti-blooming property but also acts as a cocoa butter improver to increase the heat resistance of chocolate in warm climates (Maheshwari & Reddy, 2005). Maheshwari and Reddy (2005) found that replacement of cocoa butter with 5% SOS-rich fat increased the heat-resistance property of chocolate such that it could be used as a cocoa butter improver in warm climates. Tran et al. (2015) prepared fat blends by mixing 70% cocoa butter with different SOS-rich fats and showed that the fat bloom stability and heat resistance of chocolate were improved by mixing 70% cocoa butter with 30% Vietnamese mango fat or 30% Indian mango fat stearin. However, blending of 10% fats low in SOS content with cocoa butter had little effect on fat bloom. According to Directive 2000/36/EC of the European Parliament and the Council, a maximum of 5% of finished chocolate product can be replaced by non-cocoa vegetable fats in European countries (Ray et al., 2014). Finished products with more than 5% non-cocoa vegetable fats cannot be defined as chocolate products in Europe. Therefore,

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to add SOS-rich fats within the permissible level to inhibit fat bloom or increase heat-resistance properties, pure SOS fats are preferred as an additive because the 5% permissible addition level of fats low in SOS purity cannot inhibit fat bloom effectively. Moreover, fats high in SOS content can be used alone or mixed with other fats, such as mid-fraction palm oil, to produce cocoa butter equivalent (Jin et al., 2016; Ray et al., 2014).

Theoretically, SOS can be synthesized by either enzymatic acidolysis of triolein (OOO) with stearic acid or enzymatic interesterification of triolein with stearate (Lee, Son, Akoh, Kim, & Lee, 2010; Qin, Wang, Wang, Huang, & Yang, 2011). However, the reports on enzymatic synthesis of SOS are very limited because the reaction yield of acidolysis or interesterification for the synthesis of SOS is usually low. Previously, one study reporting the synthesis of BOB by enzymatic interesterification showed that 32.8% BOB was produced in the crude mixture under optimal conditions, and the reaction yield was about 43% (Meng et al., 2013). Similar results were also reported by other researchers (Lee et al., 2010; Qin et al., 2011). Thus, rather than prepare SOS-rich fats by synthesis, researchers tend to fractionate SOS from native fats, such as kokum, shea butter and mango kernel fat, due to the low synthesis efficiency and SOS-rich fats available from natural sources. However, the availability of SOS-rich native fats from natural resources changes considerably from year to year. Furthermore, even though some native fats are rich in SOS, the separation of SOS from other types of TAGs to obtain SOS of higher than 65% purity by fractionation of native fats is still very difficult (Beckett, 2008; Jin et al., 2016; Tran et al., 2015). As a cocoa butter improver applied in the chocolate industry, SOS should have a purity higher than 75%. However, commercial SOS products of such purity are very expensive (Beckett, 2008; Jin et al., 2016). Therefore, to increase the synthesis efficiency and ensure the supply of high-quality cocoa butter improvers for the chocolate industry, there is now great interest in developing highly effective processes to produce SOS-rich fats from reliable and readily available cheap starting materials.

In this study, an effective process was developed to synthesize SOS by the enzymatic acidolysis of high-oleic sunflower oil (HOSO) with an acyl donor. The process included the enzymatic acidolysis and purification of SOS. The corresponding parameters were evaluated to maximize the yield and content of SOS among glycerides in the crude mixture. The process appears to be suitable for the large-scale production of SOS. Additionally, this methodology presents many economical and environmental advantages.

2. Materials and methods

2.1. Materials

Lipozyme RM IM (lipase from *Rhizomucor miehei*, immobilized on an anionic exchange resin), Lipozyme TL IM (lipase from *Thermomyces lanuginosus*, immobilized on silica granulation) and NS40086 (lipase from *Aspergillus oryzae*, immobilized on macroporous acrylic resin) were obtained from Novozymes (Beijing, China). Pancreatic lipase was purchased from Sigma-Aldrich Chemical Co., Ltd. (Shanghai, China). SOS, 1,2-dioleoyl-3-stearoylglycerol (SOO), methyl stearate and methyl oleate standards were purchased from Sigma-Aldrich Chemical Co., Ltd. (Shanghai, China). HOSO (882.6 g/mol of average triglyceride molecular weight), containing 87.3% oleic acid was purchased from Dow AgroSciences Co. Ltd. (Guangzhou, China). It consists of 73.7% OOO, 5.3% OOL, 6.6% OOP, 1.1% PSO, 7.8% SOO, 2.4% OLL and 1.5% POL (P = palmitic; S = stearic; O = oleic; L = linoleic). Commercial cocoa butter improver was obtained from Fuji Oil Co. Ltd. (Zhangjiagang, China). All other reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Lipase-catalyzed synthesis of SOS

HOSO (5 mmol) was mixed with an acyl donor at a controlled temperature in a three-necked round bottom flask with condensation reflux. The enzymatic reaction was conducted in either a solvent-free or solvent system. When the solvent system was applied, an organic solvent was added to the system. Lipase was used to catalyze the reaction. At the end of the reaction, the enzyme was removed by filtration, and the organic solvent was evaporated under reduced pressure. The acyl donor and lipase were screened first, and then four parameters, including solvent quantity, substrate molar ratio of acyl donor to HOSO, reaction temperature and time, were optimized to maximize the yield and content of SOS among the glycerides.

The examined acyl donors included stearic acid, methyl stearate and vinyl stearate, and the selected lipases were Lipozyme TL IM, Lipozyme RM IM and NS40086. Solvent quantity ranged from 0 to 6 ml; substrate molar ratio (stearic acid/HOSO) ranged from 4 to 16; heating temperature was set in the range of 70 to 80 °C; and reaction time was varied within the range of 2 to 8 h. When the reaction conditions were optimized, one factor was changed at each level, and the other factors were kept at a fixed level. After one of the factors was optimized, the optimal value of this factor was used for the next factor optimization. All reactions were run in duplicate unless otherwise specified. The data are presented as means ± standard error of mean (SEM) of two separate experiments.

2.3. Purification of crude SOS product

The crude SOS product was purified in two steps: separation of SOS from free fatty acids (FFAs) by molecular distillation and further separation of SOS from other TAG species by solvent fractionation. First, FFAs were removed by molecular distillation, based on their differences in volatility. The parameters of the process were set as follows: distillation temperature of 205 °C; rotation speed of the wiped film of 120 rpm; feed speed of 2 ml/min; vacuum pressure of 8 Pa; preheating temperature of 80 °C and condensate temperature of 65 °C. The product was separated into two phases under these conditions. The heavy phase contained TAGs, and FFAs were distilled out as the light phase. The FFA-removed product was collected for further purification.

In a further study, solvent fractionation was used to purify SOS; the FFA-removed product was blended with acetone, and the mixture was subsequently placed at a controlled temperature for a certain period of time after being heated at 55 °C for 5 min. At the end of the fractionation, the solid and liquid fractions were separated by filtration to obtain SOS-rich fat in the solid fraction. Fractionation temperature, time and the substrate ratio of acetone to semi-purified TAGs (v/w) were evaluated until optimization of the process was achieved. The fractionation temperature was set in the range of 5 to 25 °C; fractionation time ranged from 1 to 5 h; and the substrate ratio was in the range of 1 to 7.

2.4. Analysis

2.4.1. Analysis of TAG composition by HPLC

For the separation and quantification of the reaction and fractionated products, reversed-phase HPLC-ELSD, using a Waters 1525 liquid chromatographic system (Waters Corp., Milford, MA, USA) equipped with a Sunfire C18 column (150 × 4.6 mm, Waters Corp.), was chosen, and the products were eluted with a binary gradient of solvent A (100% acetonitrile) and solvent B (1:1, iso-

propanol/hexane, v/v) at 1.0 ml/min. Samples were diluted to a concentration of 1 mg/ml and analyzed according to the following gradient profiles: solvent A was decreased from 60% to 40% over 10 min and increased further to 60% from 10 to 20 min. Lastly, solvent A was held at 60% for 5 min. The total run time was 25 min. The ELSD temperature was set to 55 °C at a gain of 5, and the flow rate of high-purity nitrogen was set to 1.8 ml/min. SOS and SOO were used as external standards to identify the peaks based on the HPLC retention times.

2.4.2. Analysis of sn-2 fatty acid composition

Because separation of TAGs on reversed-phase HPLC-ELSD is based on the equivalent carbon number, SOS cannot be separated from 1,2-distearoyl-3-oleoylglycerol (SSO) by this method and it will appear together with SSO as one peak. To confirm SOS regiopurity (*sn*-SOS purity), the fraction containing SOS and SSO was first prepared by preparative HPLC. Subsequently, the collected fraction was used for the quantification of SOS and SSO positional isomers by *sn*-2 fatty acid composition analysis. The method for the preparation of SOS and SSO fraction is the same as that for the reversed-phase HPLC mentioned above, except that a Sunfire C18 column (150 × 19 mm, Waters Corp.) and flow rate of 8.0 ml/min were used.*sn*-2 fatty acid composition was analyzed, based on a previous method (Nagachinta & Akoh, 2013). Namely, 1 ml of 1 M Tris-HCl buffer (pH 8.0), 0.1 ml of 2.2% $CaCl_2$, 10 mg pancreatic lipase and 0.25 ml of 0.05% bile salts were mixed with an SOS fraction containing *sn*-SOS and *sn*-SSO. The mixture was incubated in a water bath at 39 °C for 3 min with shaking, and then 1 ml of 6 M HCl solution and 2 ml of diethyl ether were added and centrifuged. The diethyl ether was then dried by anhydrous sodium sulfate. The product was separated on thin-layer chromatography plates, and the developing solvent was hexane/diethyl ether/acetic acid (50:50:1; v/v/v). The band corresponding to 2-monoacylglycerols (2-MAGs) was scraped off and analyzed as described below.

For the preparation of fatty acid methyl esters of 2-MAGs, around 20 mg of 2-MAGs were mixed with 2 ml of 0.5 M NaOH-CH_3OH solution at 60 °C for 30 min. The resulting product was reacted with 14% boron trifluoride-methanol solution at 65 °C for 5 min, and then 3 ml of hexane was added to the mixture to extract the fatty acid methyl esters. Methyl stearate and oleate were used as external standards, and the peaks were identified, based on the gas chromatography retention times.

Fatty acid methyl esters were identified and quantified, using a GC-14B (Shimadzu, Tokyo, Japan) equipped with a flame ionization detector, using a 30 m × 0.25 mm × 0.25 μm (length × I.D. × film thickness) fused-silica capillary column PEG-20000. The oven temperature was programmed from 110 to 250 °C at a rate of 8 °C/min and held at 250 °C for 10 min. The injector and detector temperatures were set at 250 °C.

2.5. Statistical analysis

All data were analyzed by one-way ANOVA. Differences among the means were compared at $P = 0.05$, using Tukey's test. Different letters in the figures indicate significant differences for the specific quality parameter.

3. Results and discussion

3.1. Screening of acyl donor and lipase

SOS was synthesized by either acidolysis of HOSO with stearic acid or interesterification of HOSO with methyl stearate or vinyl stearate. The acyl donor affects the substrate interaction and may influence the reaction efficiency. The results of the effect of the acyl donor are presented in Fig. 1. They showed that the acyl donor significantly influenced the SOS yield. For all three lipases, when vinyl stearate was used as the acyl donor, SOS yield was significantly lower than that obtained using stearic acid and methyl stearate as the acyl donors, regardless of the lipases used. A yield below 10% was achieved with vinyl stearate as the acyl donor. No significant differences were observed in SOS yield between stearic acid and methyl stearate. When stearic acid was used as the acyl donor, SOS yield was obtained in the range of 24% to 50.6%, whereas the use of methyl stearate led to SOS yield in the range of 29% to 49%. Because stearic acid is a readily available, cheap starting material, it was used as the acyl donor for further experiments.

Previous studies have reported that fatty acid vinyl ester has advantages over other acyl donors in the transesterification reaction (Halldorsson, Magnusson, & Haraldsson, 2003; Tang, Wang, Huang, Jin, & Wang, 2015). Transesterification with vinyl ester as the acyl donor is highly effective because the reaction of fatty acid vinyl ester with the –OH group of the partial glyceride results in the formation of non-nucleophilic acetaldehyde. Acetaldehyde has quite a low boiling point (20.8 °C) and will immediately evaporate at room temperature, making the transesterification irreversible (Tang et al., 2015). However, when fatty acid vinyl ester is used in interesterification, the reaction is very different because there is no –OH group available for the formation of non-nucleophilic acetaldehyde. Therefore, the use of vinyl ester in interesterification for the synthesis of SOS is not feasible.

The selected lipases used for the screening of lipase included Lipozyme RM IM, NS40086 and Lipozyme TL IM. All are *sn*-1,3-specific lipases rather than non-specific lipases, such as Novozym 435 and Lipozyme 435, because non-specific lipases will cause randomization of SOS to form many other TAGs, such as SSS, SOO and OOO. As shown in Fig. 1, the type of lipase also had a significant effect on the interesterification of HOSO. With stearic acid and methyl stearate as the acyl donors, Lipozyme RM IM and NS40086 showed higher catalytic activities than did Lipozyme TL IM. However, no significant differences in SOS yield were found between Lipozyme RM IM and NS40086. When stearic acid was chosen as the optimal acyl donor, the NS40086-catalyzed reaction resulted in the formation of SOS at a 50.6% yield, whereas a 38.7% yield was achieved with the Lipozyme RM IM-catalyzed reaction. Based on these results, NS40086 was selected as the optimal lipase for further reactions.

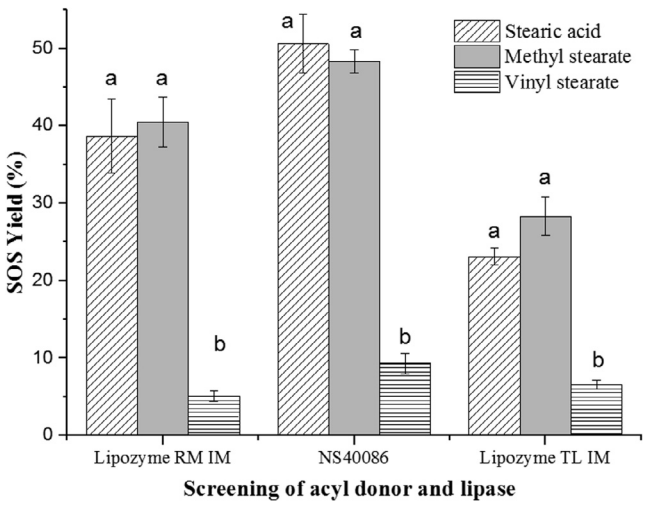

Fig. 1. Screening of the acyl donor and lipase. Reaction conditions: substrate molar ratio of 6 (acyl donor/HOSO), reaction temperature of 70 °C, reaction time of 6 h, lipase loading of 10% and a solvent-free system.

Generally, the most common *sn*-1,3-specific lipase for the synthesis of symmetrical TAGs is Lipozyme RM IM. However, Lipozyme RM IM has low thermal stability. Reactions conducted at temperatures above 60 °C have an adverse effect on the catalytic activity of Lipozyme RM IM (Haar, Stäbler, Wichmann, & Schweiggert-Weisz, 2015). Therefore, the optimal reaction temperature in interesterification and acidolysis reactions is usually not higher than 65 °C (Sahin, Akoh, & Karaali, 2005; Wang et al., 2015). The present study also showed that, when the reaction temperature of Lipozyme RM IM-catalyzed acidolysis was carried out at 70 °C, the catalytic activity of Lipozyme RM IM in acidolysis was limited and decreased at higher temperatures. In contrast, NS40086 exhibited good thermal stability in the range of 70 to 80 °C as shown below. NS40086 is a new lipase produced by Novozymes. To date, to our knowledge, no publication has reported its applications in the synthesis of structured lipids. The present study showed that NS40086 had a better stability at high temperatures than did Lipozyme RM IM and may have wider applications in lipid synthesis.

3.2. Optimization of SOS synthesis

Generally, optimization of synthesis aims to maximize the yield of the target product, and only this yield is considered when selecting reaction conditions (Lee et al., 2010; Meng et al., 2013). In this study, in addition to considering the yield of target product SOS, we also considered the content of monostearoyl-containing TAGs (mainly SOO) in the final product when selecting the optimal conditions, because the separation of SOS from monostearoyl-containing TAGs is more difficult than that from FFAs and HOSO. SOS and FFAs have large differences in volatility. Thus, FFAs can be easily removed by molecular distillation, whereas SOS can be easily fractionated from HOSO (73.7% OOO) due to their differences in melting points. The differences in melting point (or volatility) are larger between SOS and HOSO (or FFAs) than between SOS and SOO. Therefore, the amount of monostearoyl-containing TAGs in the crude mixture will affect final purity of SOS after purification.

First, the effect of an additional amount of hexane on interesterification of HOSO with stearic acid was investigated as it may improve the interaction between HOSO and stearic acid. In addition, the presence of hexane in the reaction system can reduce the viscosity of the system, which may be beneficial for substrate interaction. The results of the effect of hexane addition are presented in Fig. 2a. This shows that when the hexane quantity was changed from 0 to 6 ml, yield did not change significantly and SOS was obtained in a yield of around 50%. The insignificance of these results might be caused by a dual effect of hexane addition on interesterification. The presence of solvent improves the substrate interaction and reduces viscosity, which has a positive effect on SOS yield; however, the addition of solvent also dilutes the lipase concentration, which causes a reduction in lipase catalytic efficiency. Because the use of hexane, not only increases the cost, but also has a potentially adverse effect on health, a solvent-free system is the preferred choice in this study.

Second, the effect of the substrate molar ratio of stearic acid to HOSO on the interesterification of HOSO was investigated because it affects conversions of HOSO and SOO to the target product SOS. The conversion of SOO to SOS during interesterification is particularly important because the separation of SOO from SOS is difficult, and the quantity of SOO in the interesterified mixture will determine SOS purity in the final product. The results in Fig. 2b show that the SOS yield increased as the substrate molar ratio increased. When the substrate molar ratio was varied from 4 to 16, SOS yield ranged from 43.8% to 73.6%, whereas it was not affected by substrate molar ratios ranging from 10 to 16. However, interesterification conducted at a molar ratio of 10 tended to significantly increase SOS yield over that at a molar ratio of 8 ($P = 0.06$). Even though SOS yield obtained at a molar ratio of 10 was not significantly different from that obtained at a molar ratio of 12, the molar ratio of 12 resulted in a lower SOO content among the glycerides. Therefore, a molar ratio of stearic acid to HOSO of 12 was used as the optimal value for further interesterifications. Under these conditions, SOS was obtained in a 72.5% yield.

In previous studies reporting the synthesis of anti-blooming BOB, a low substrate molar ratio usually was used (Meng et al., 2013; Padley, 1996). However, use of a low molar ratio resulted in an incomplete reaction, and large quantities of monobehenoyl-containing TAGs were formed in the crude product. For example, Padley (1996) performed lipase-catalyzed acidolysis of rapeseed oil with a mixture of fatty acids containing behenic acid at a low substrate molar ratio. At the end of the reaction, the product contained 19.5% of desirable dibehenoyl-containing and 44.7% of undesirable monobehenoyl-containing TAGs. Meng et al. (2013) conducted enzymatic interesterification of HOSO with behenic acid methyl ester for the synthesis of BOB at a low substrate molar ratio and obtained a target product BOB content of 32.8%, whereas a BOO content of up to 34.4% was present in the crude product. Nevertheless, we do not think that a low molar ratio is an optimal condition because, when the researchers optimized the effect of the substrate ratio, the BOB content increased in line with the substrate ratio. The increasing substrate ratio did not show a tendency for the BOB content to decrease. In this situation, the effects of higher substrate ratios should have been investigated, but this was not done. In the present study, a higher substrate ratio was beneficial for the conversions of HOSO and SOO to SOS, and this agrees with a previous study using a high substrate ratio for the synthesis of structured lipids (Sahin et al., 2005).

Subsequently, the effect of reaction temperature was examined because temperature affects reaction rate, substrate interaction and lipase activity. Heating temperature correlates positively with reaction rate and substrate interaction. Differently from the reaction rate and substrate interaction, heating temperature had a dual effect on lipase activity. Low reaction temperatures are not beneficial for increasing the catalytic activity of lipase, but excessively high temperatures denature the proteins in lipase, resulting in its deactivation. The results of temperature optimization are presented in Fig. 2c. An initial temperature of 70 °C was selected to study the effect of temperature because stearic acid has a melting point of about 70 °C and it solidifies during interesterification at a temperature below 70 °C. As shown in Fig. 2c, temperatures in the range of 70 to 80 °C did not significantly affect SOS yield, whereas a temperature higher than 80 °C significantly influenced the lipase activity. However, NS40086 lipase showed good catalytic activity at 70 and 75 °C. Based on these results, 70 °C should be selected as the optimal temperature. However, the glycerides produced in the reaction conducted at 70 °C contained 23.4% of monostearoyl-containing TAGs (mainly SOO), whereas those produced in the reaction conducted at 75 °C contained 19.8% of monostearoyl-containing TAGs. To reduce the content of monostearoyl-containing TAGs in the crude mixture to its lowest level, 75 °C was used for the optimization of reaction time.

Finally, reaction time was optimized. As shown in Fig. 2d, reaction time had a positive effect on SOS yield when time was changed within the range of 2 to 8 h. However, with the increase in reaction time from 4 to 8 h, the elevation of SOS yield was slight and insignificant. Thus, 4 h was chosen as the optimal reaction time. Under these optimized conditions (10% NS40086 as catalyst, a solvent-free system, substrate ratio of 12 and 75 °C for 4 h), SOS was obtained in a yield of 70.2%. After the optimization, the glycerides of the crude acidolysis product were composed of 68.3% SOS, 7.4% SSO, 13.5% SOO, 3% OOO and 4.9% partial glycerides.

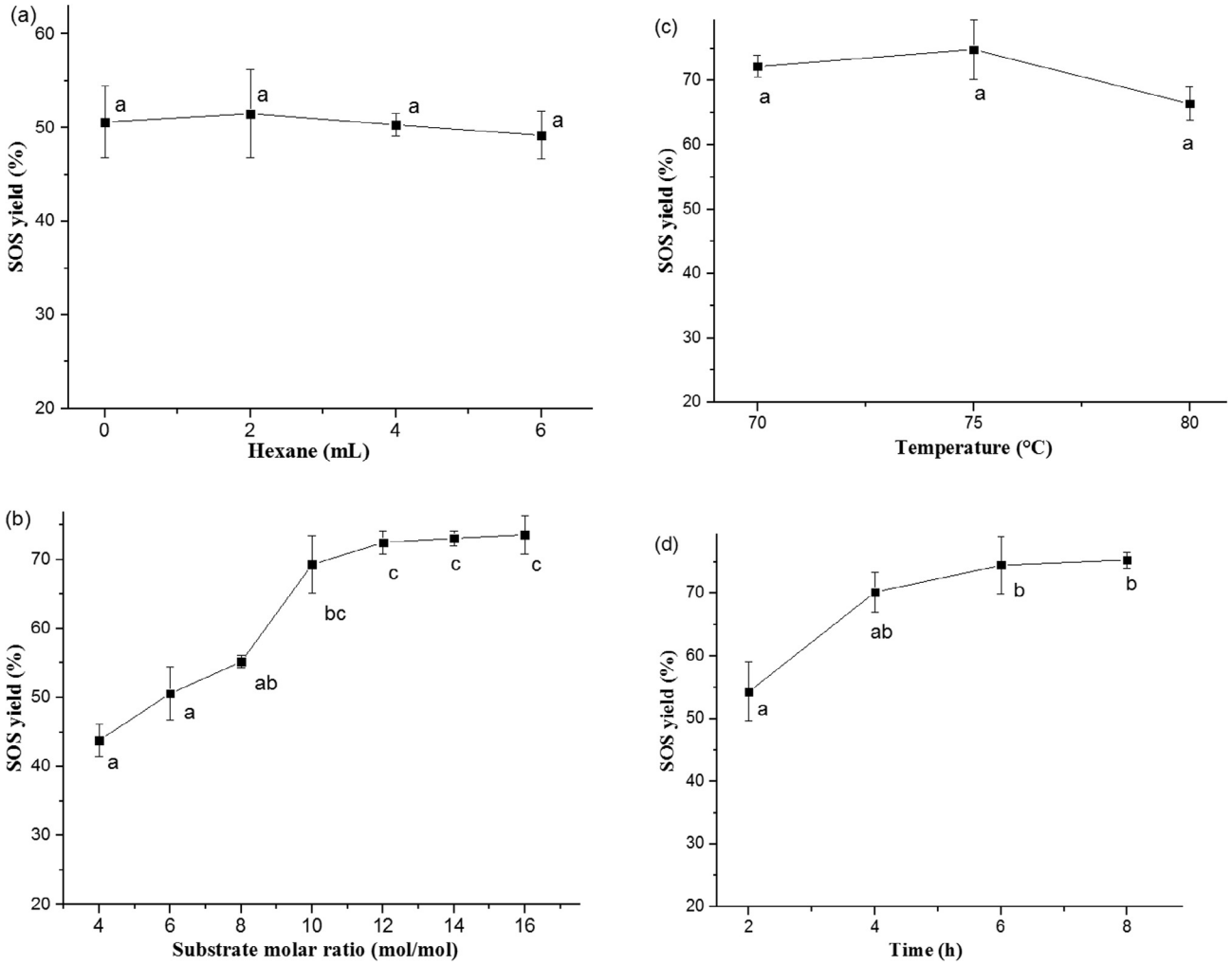

Fig. 2. Optimization of reaction conditions for SOS synthesis by enzymatic acidolysis of HOSO with stearic acid. Reaction conditions: (a), substrate molar ratio of 6 (stearic acid/HOSO), reaction temperature of 70 °C, reaction time of 6 h and NS40086 loading of 10%; (b), reaction temperature of 70 °C, reaction time of 6 h, NS40086 loading of 10% and a solvent-free system; (c), substrate molar ratio of 12, reaction time of 6 h, NS40086 loading of 10% and a solvent-free system; (d), substrate molar ratio of 12, reaction temperature of 75 °C, NS40086 loading of 10% and a solvent-free system.

3.3. Two-step purification of crude SOS product

The SOS was purified in two steps. First, FFAs were removed from the TAGs by molecular distillation, due to their differences in volatility. After molecular distillation, the content of FFAs in the heavy phase was 0.16% and that of the TAGs in the light phase was undetectable, suggesting that the FFAs were fully removed by molecular distillation.

Second, the FFA-removed product was purified further to separate SOS from HOSO and monostearoyl-containing TAGs by acetone fractionation. The FFA-removed product contained 70.4% SOS, 22.3% monostearoyl-containing TAGs, 3.7% unreacted HOSO and 2.2% partial glycerides among the total glycerides. Partial glycerides contain an –OH group and a very small amount of partial glycerides that are soluble in acetone, whereas SOS solidifies out from acetone at a low temperature. In addition, HOSO has quite a low melting point of around 4 °C and will not crystallize out in an acetone solution at the selected temperatures. Therefore, partial glycerides and HOSO can be readily removed by acetone fractionation.

The effects of fractionation temperature, time and the ratio of acetone to FFA-removed product on SOS content and recovery were investigated, and the results are given in Fig. 3. The results in Fig. 3a reveal that SOS content increased with elevation of the temperature in the range of 5 to 20 °C and decreased with elevation of the temperature from 20 to 25 °C. In contrast, SOS recovery followed an inverse relationship, with temperature in the range of 5 to 25 °C. When fractionation was conducted at a low temperature, almost all of the SOS was crystallized out, thus resulting in increased SOS recovery. However, monostearoyl-containing TAGs were also crystallized out at such a temperature, thereby reducing SOS purity. With the increase in fractionation temperature from 5 to 20 °C, SOS purity increased as more and more monostearoyl-containing TAGs became liquid and separated from the SOS crystal, indicating increased SOS purity and decreased recovery. However, when the fractionation was performed at 25 °C, most of the SOS was liquid. Taking both SOS purity and recovery into account, 15 °C was selected as the optimal temperature. Under these fractionation conditions, 88.7% of SOS was obtained with an 86.7% recovery. In addition, the fractionation product contained 7.5% SOO and 1.9% POS.

Next, the effects of the ratio of acetone to FFA-removed product (v/w) were studied; the results are presented in Fig. 3b. When the ratio was changed from 1 to 7, SOS purity increased slightly with the increase in ratio. However, the ratio of acetone to FFA-removed product did not significantly affect SOS purity. SOS recov-

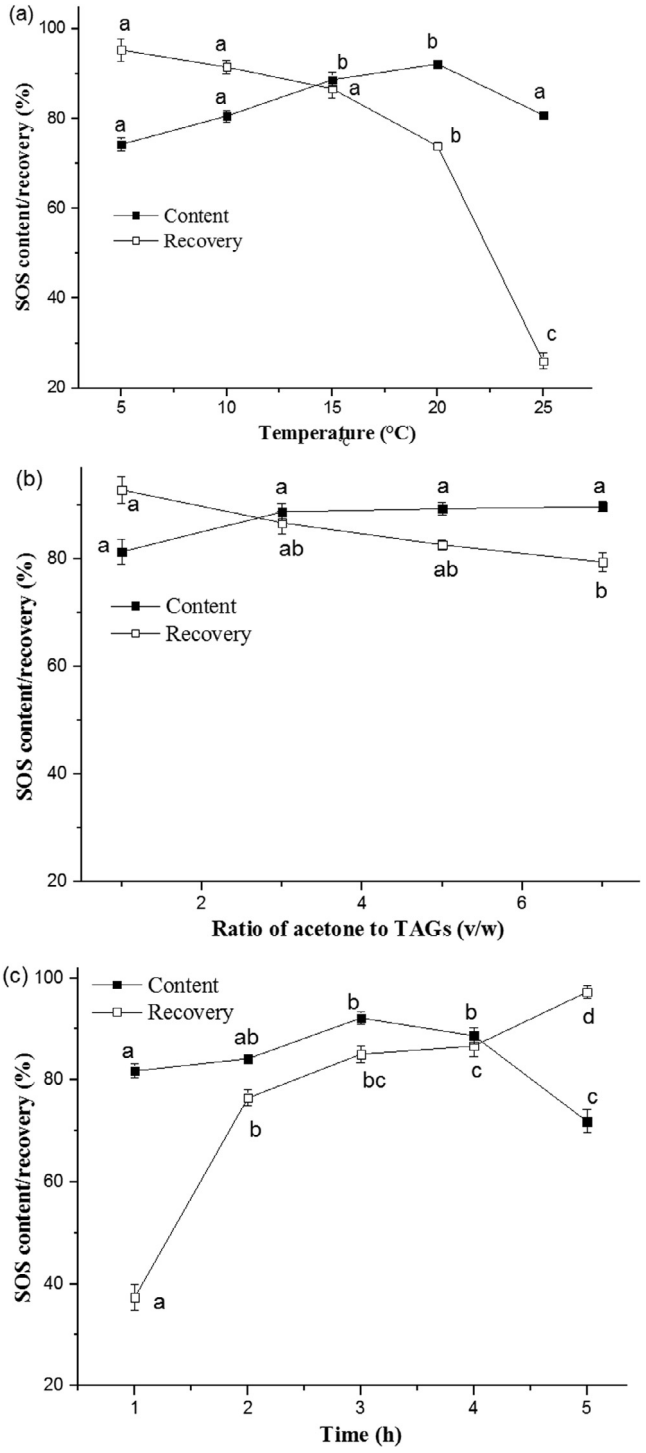

Fig. 3. Optimization of fractionation conditions for SOS purification. Reaction conditions: (a), a ratio of 3 of acetone to the FFA-removed product (v/w), fractionation time of 4 h; (b), fractionation temperature of 15 °C, fractionation time of 4 h; (c), fractionation temperature of 15 °C, a ratio of 3 of acetone to TAGs.

ery decreased slightly as the ratio increased; it was significantly lower at a ratio of 1 than at a ratio of 7. The increased ratio caused more TAGs to solubilize in acetone, suggesting reduced recovery. Nevertheless, the increase in the ratio had limited effect on SOS purity, which remained constant at a range of 3 to 7. Taking both SOS purity and recovery into account, a ratio of 3 was selected for further fractionation. Under these conditions, the fractionation product contained 88.7% SOS, 7.5% SOO and 1.9% POS.

Lastly, the effects of fractionation time on SOS purity and recovery were investigated. The results presented in Fig. 3c indicate that fractionation time correlated positively with SOS recovery as more TAGs were crystallized out from the solution with time. Differently from SOS recovery, SOS purity showed an increasing-decreasing pattern over the range of 1 to 7 h. It increased at the beginning of fractionation due to the crystallization of SOS but, as time passed, the monostearoyl-containing TAGs started to crystallize, resulting in reduced SOS purity. Taking both SOS purity and recovery into account, 3 h was chosen as the optimal fractionation time. Therefore, the optimal fractionation conditions were a fractionation temperature of 15 °C, a ratio of 3 of acetone to FFA-removed product and a fractionation time of 3 h. Under these conditions, 92.2% SOS was obtained with an 85.1% recovery. The TAG profile is given in Table 1.

Because HPLC cannot separate *sn*-SOS from *sn*-SSO, fractionated SOS contained two isomers: *sn*-SOS and *sn*-SSO. To determine the regiopurity of SOS, pure SOS was first prepared by preparative HPLC. Subsequently, the *sn*-2 fatty acid composition of pure SOS was measured, which showed that 90.3% oleic acid and 9.7% stearic acid were located in the *sn*-2 position of SOS, suggesting that SOS contained 90.3% *sn*-SOS and 9.7% *sn*-SSO. When Lipozyme RM IM was used as a catalyst, pure SOS prepared by preparative HPLC barely contained any *sn*-SSO (data not shown). The results indicated that, compared to Lipozyme RM IM, NS40086 exhibited higher catalytic activity in acidolysis and lower selectivity toward *sn*-1,3 positions of the TAGs. The high *sn*-SSO content in SOS may also be attributed to a high reaction temperature of 75 °C, which accelerates migration of oleic acid from the *sn*-2 to *sn*-1,3 position in the presence of a lipase.

To compare physicochemical properties of synthetically purified SOS product with a commercial cocoa butter improver product, various properties of these two products, such acid value, peroxide value, iodine value, saponification value and TAG composition were measured. The data can be compared in Table 1. The results showed that the physicochemical properties of the synthetic SOS product were similar to those of the commercial product. Although both had similar SOS contents, the commercial product contained a lower *sn*-SSO content than the synthetic SSO product. In contrast, the *sn*-POS content present in the commercial product was higher. Based on this analysis, the synthetic SOS product after purification can be used as a cocoa butter improver. Additionally, the physical properties of blends of cocoa butter with fractionated SOS were also studied. The ratios of cocoa butter to fractionated SOS were set in the range of 100% to 0%. The results are presented in Fig. 4. Overall, the solid fat content increased with increase in fractionated SOS content in the mixture. When fractionated SOS was blended with cocoa butter in ratios ranging from 20% to 80%, good compatibility was observed between them (Fig. 4).

Table 1
Comparison of physicochemical properties of synthetic SOS and commercial cocoa butter improver products[a].

Parameters	Synthetic SOS product	Commercial product
Acid value (mg KOH/g oil)	0.11	0.07
Peroxide value (mmol/kg)	0.3	0.5
Iodine value (g I/100 g oil)	32.9	35.1
Saponification value (mg/g)	199.8	194.2
TAG composition (%)		
sn-SOS	83.3	81.2
sn-SSO	8.9	Undetectable
sn-SOO	5.5	9.1
sn-POS	1.2	7.4
sn-SSS	0.6	0.7
sn-OOO	0.5	1.6

[a] *sn*-SOS content = 92.2% × 90.3% = 83.3%; *sn*-SSO content = 92.2% × 9.7% = 8.9%.

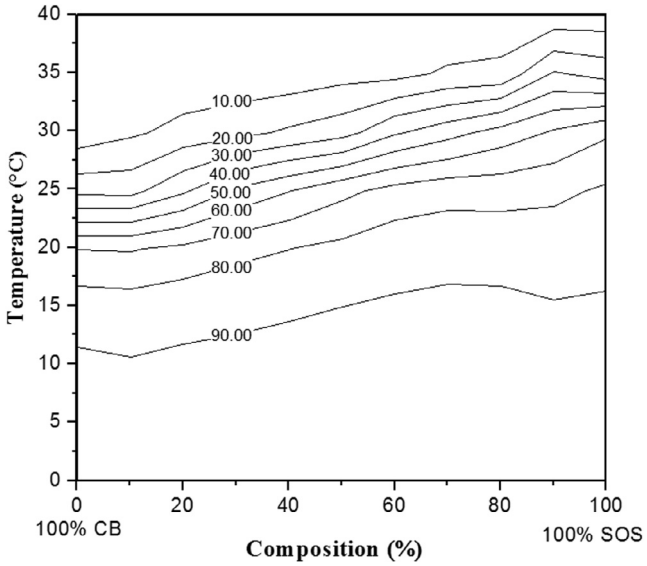

Fig. 4. Binary isosolid phase diagrams for different mixtures of cocoa butter and fractionated SOS.

4. Conclusion

A high-quality SOS product is extremely expensive, and the high cost of SOS as a cocoa butter improver limits its availability and application in the chocolate industry. In this study, a highly effective acidolysis was developed for the production of SOS. Compared with previous studies synthesizing TAGs by enzymatic acidolysis, yield of the target product was significantly improved. This result can be explained by the use of a high substrate ratio and NS40086 instead of Lipozyme RM IM. Although NS40086 had a higher tolerance of high temperature, its selectivity toward *sn*-1,3 positions was lower than that of Lipozyme RM IM. After two-step purification, the synthetic SOS product exhibited physicochemical properties similar to those of a commercial product, suggesting that it can be used as a cocoa butter improver or anti-blooming agent.

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