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Enzymatic synthesis of cocoa butter analog through interesterification of lard and tristearin in supercritical carbon dioxide by lipase

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Abstract

Substrate oil composition, reaction time, acyl donor, temperature, and pressure affected the triacylglycerol (TG) content of cocoa butter analog during the interesterification reaction catalyzed by lipase in a supercritical carbon dioxide (SC-CO_2) system. Among oil sources used to interact with tristearin, the content of 1(3)-palmitoyl-3(1)-stearoyl-2-monoolein (POS) (P, palmitate; O, oleate; S, stearate) and 1-palmitoyl-2, 3-dioleoylglycerol (POO) in analog was most similar to the corresponding TG content of cocoa butter when analog was prepared with lard. The optimized interesterification reaction using lard and tristearin (at a mole ratio of 1.4) as substrates to produce cocoa butter analog in a SC-CO_2 system was at 17 MPa, 50 °C, pH 9, for 3 h with an immobilized lipase, Lipozyme IM-20, from *Mucor miehei*. The lyophilized enzyme facilitated the production of cocoa butter analog in anhydrous substrates (a_w 0.33). The yield and melting point of the purified cocoa butter analog by a silica column was 63% and 34.5 °C, respectively, when the analog was produced under optimal conditions.

Keywords: Lipase; Cocoa butter analog; Lard; Interesterification reaction; Supercritical carbon dioxide

1. Introduction

Cocoa butter is characterized with sharp melting point, desirable physicochemical properties, and fatty acid components (Shukla, 1996), and is melted fast and completely in mouth while it is hard and brittle at room temperature. It is an important major constituent of the chocolate formulations and is composed predominantly (>70%) of symmetrical triacylglycerols, 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1(3)-palmitoyl-3(1)-stearoyl-2-monoolein (POS), and 1,3-distearoyl-2-oleoylglycerol (SOS), with oleic acid in the sn-2 position. The typical fatty acid composition of cocoa butter in mole percentage is: $C_{16:0}$ 24.4%, $C_{18:0}$ 33.6%, $C_{18:1}$ 37.0%, $C_{18:2}$ 23.4% and others 1.6% (Shukla, 1996). Cocoa butter melts between 32 and 35 °C and is able to re-crystallize during processing to a stable crystal mode. However, due to the uncertainty in supply and the volatility in prices, some trials have been made to seek alternatives for genuine cocoa butter.

Procedures of chemical interesterification for analogs have been developed for years from cheap fats and oils

Abbreviations: POP, 1,3-dipalmitoyl-2-oleoylglycerol; POS, 1(3)-palmitoyl-3(1)-stearoyl-2-monoolein; POO, 1-palmitoyl-2,3-dioleoylglycerol; SOS, 1,3-distearoyl-2-oleoylglycerol; SOO, 1-stearoyl-2,3-dioleoylglycerol.

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and are found to be practical to industries. Recently, due to the high specificity and efficiency in acyl exchange reaction, lipases are becoming important in interesterification reaction in producing analogs and some cheap commercial fats and oils such as beef tallow, lard, rapeseed oil, and sunflower oil have been converted into high value-added products by enzymatic interesterification (Forssell et al., 1992). Moreover, vegetable oils such as Mahua, kokum and mango fats, fatty acid methyl ester, palm oil midfraction, teaseed oil, stearic acid, palmitic acid, and olive oil have been popularly used to prepare cocoa butter equivalents (CBE) by lipases in batch stirred tank reactor, orbital shaker or vessel in recent decades (Chang, Abraham, & John, 1990; Macrae, 1983; Sridhar, Lakshminarayana, & Kaimal, 1991; Wisdom, Dunnil, & Lilly, 1987). Currently, mixture of substrate oil and free fatty acid is passed through a packed enzyme bed, followed by distillation, evaporation, fractionation, and refining processes to produce CBE in food industry. However, CBE prepared through the lipase-catalyzed interesterification of triacylglycerols in organic solvent systems (Chang et al., 1990; Macrae, 1983; Sridhar et al., 1991; Wisdom et al., 1987) are suffering from some major drawbacks such as final solvent removal, which is usually costly and lengthy. Supercritical fluids, with less mass transfer resistance than the conventional liquid solvents, have been used as solvents for enzymatic reactions (Hammond, Karel, & Klibanov, 1985; Pasta, Mazzola, Carrea, & Riva, 1989; Randolph, Blanch, & Prausnitz, 1998). In addition, the solubility of most organics in supercritical fluids is higher than that in gaseous phase and is comparable with liquid solvents. Among supercritical fluids, SC-CO_2 is especially advantageous due to its low price, non-toxicity, non-inflammability, negligible environmental impact, near-ambient critical temperature, and moderate critical pressure (Novotny, Bertsch, & Zlatkis, 1971; Randolph et al., 1998).

Interesterification and transesterification reactions of fats and oils by lipases using SC-CO_2 as medium have been tried frequently (Nakamura, Chi, Yamada, & Yano, 1985; Pasta et al., 1989; Randolph et al., 1998). Taking the advantage of low solubility of enzyme and cocoa butter analog product in carbon dioxide at room temperature, depressurization of reactor after reaction enhances the separation of product from reaction mixture and reduces the production cost. Liu and Shaw (1997) indicated that interesterification of palm oil and tristearin to produce CBE by lipase in a SC-CO_2 system was far efficient and economic than that in *n*-hexane system.

Higher contents of POS, SOS, and SOO in lard than in palm oil may facilitate the formation of cocoa butter analog. Besides, reports on producing CBE with lard or tallow are rare (Zhang, Hu, & Xu, 1994). In the present work, a trial on the formation of cocoa butter analog from lard and tristearin by interesterification reaction catalyzed by lipase in a SC-CO_2 system was done. In the first phase of the present research, formation of cocoa butter analog versus lipase source was investigated. Then, triacylglycerol (TG) content of cocoa butter analog versus oil type, reaction time, acyl donor, reaction temperature, pressure, lard/tristearin ratio, water content, and pH value were also studied. Finally, yield of cocoa butter analog by interesterification of lard or palm oil with tristearin in a SC-CO_2 system with lipase was compared.

2. Materials and methods

2.1. Materials

Lipases from *Aspergillus niger*, *Penicillium camembertii* (Amano "G") and *Pseudomonas cepacia* (Amano "PS") were purchased from Amano International Enzyme Co. (Nagoya, Japan), while lipase from *Cadida cylindracea* Type VII, stearic acid, stearic acid ethyl ester, stearic anhydride, and tristearin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Immobilized lipase, Lipozyme IM-20, from *Mucor miehei* was the product of Novo Nordisk Inc. (Danbury, CT, USA). *n*-Hexane, *t*-butyl alcohol, and ethanol were obtained from Merck Chemical Co., Darmstadt, Germany. POP, POS, POO (1-palmitoyl-2,3-dioleoylglycerol), SOS and SOO (1-stearoyl-2,3-dioleoylglycerol) were purchased from Matreya Inc., Pleasant Gap, PA, USA. Palm oil and cocoa butter were purchased from First Chemical Co., Taipei, Taiwan. Refined, bleached and deodorized lard, containing 102 mg/100 g cholesterol (Lanzanl et al., 1994), was purchased from a local supermarket (Kaohsiung, Taiwan). Carbon dioxide with a purity of 99.99% was from Yun-Shan-Hang Co., Tainan, Taiwan.

2.2. Cocoa butter analog synthesis in SC-CO_2 system

Cocoa butter analog synthesis was carried out in a SC-CO_2 batch reactor, a 40 mL-high pressure vessel. Into which, 90 mg immobilized lipase (Lipozyme IM-20), 0.06 mmol (55 mg) lard, 0.02 mmol (30 mg) tristearin, and 5 μL water were loaded to conduct the interesterification reaction at 17 MPa psi and 50 °C for 3 h. After reaction to the desired time, depressurization and elution by *n*-hexane was conducted, followed by extraction twice with diethyl ether to remove free fatty acids. The thus obtained *n*-hexane layer was concentrated with a rotary evaporator (R200A, Büchi, Switzerland) (30 °C, 80 rpm) at a reduced pressure of 50 Torr to obtain the dried solid, which was then heated in a oven at 37 °C for 30 min to collect the liquid portion by centrifugation (2000*g*, 37 °C, 5 min). The excessive lard and tristearin in reaction mixture were all removed during centrifugation.

Subsequently, 2 g of thus obtained liquid was suspended homogenously in 10 mL of mixture of *n*-hexane and diethyl ether (1:1, v/v), followed by application to a Silica column (2.0 × 25 cm), packed with ASTM silica gel 60 (70–230 mesh) and elution by 750 mL of the same *n*-hexane–diethyl

ether solvent. Fractionation of 50 mL/tube was collected for the following gas chromatography (GC) analysis described below.

2.3. Optimization of cocoa butter analog formation

Experiments were conducted to study the factors, such as enzyme source, oil source, reaction time (1–7 h), reaction pressure (10, 17, and 24 MPa), acyl donor source, reaction temperature (40–60 °C), pH value (pH 3–10), and mole ratio (8.6–1.0) of lard to tristearin (lard/tristearin), affecting the TG content of cocoa butter analog. First, effects of lipase type, oil type, and various reaction times on triacylglycerol (TG) content of cocoa butter analog were studied under the same conditions except the pressure used was 10 MPa. Effect of reaction pressure on TG content of analog was investigated under the same experimental conditions except that the pressure was set at 10, 17, and 24 MPa. Similarly, acyl donors from various sources, various reaction temperatures, various pH values, and various mole ratios (8.6–1.0) of lard to tristearin (lard/tristearin) were applied to the reactor to study their effects on the TG content of analog under the same conditions. To study the effect of water content on content of each TG (POP, POS, POO, SOS, and SOO), various amounts (0–25%, v/v) of distilled water were added to the Lipozyme IM-20-lard-tristearin mixture and the experiments were conducted under the same conditions.

To control precisely the water content in the reaction mixture, lipases used were previously lyophilized by a Savant Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY) under 50 mTorr for 24 h, while moisture in *n*-hexane, used to perform interesterification, was previous removed by 3 Å molecular sieve (CAS number 1318-02-1, Merck KGaA Chemical Co., Darmstadt, Germany). To study the pH effect on analog synthesis, lipase was dissolved in 10 mM mixed Good's buffer solution containing 10 mM each of *N*,*N*-bis(2-hydroxyethyl)-glycin,3-(cyclohexylamino)-1-propane-sulfonic acid, sodium acetate, and 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Good et al., 1966), followed by pH adjustment to pH 3–10 by adding concentrated HCl or 2 N NaOH solution and lyophilization by a Savant Speed Vac Concentrator. The "Good" zwitterionic buffers, by definition, contain both positive and negative ionizable groups. Secondary and tertiary amines provide the positive charges, while sulfonic and carboxylic acid groups provide the negative charges.

2.4. GC analysis

Pooled fraction (1.0 μL) of TG from a Silica column was applied to a GC analyzer (Model G-3000, Hitachi, Tokyo, Japan) using a Rtx®-65TG fused-silica capillary column (30 m × 0.25 mm, inner diameter) (Restek Corporation, Bellefonte, PA). The chromatographic conditions were as follows: carrier gas, H_2 (1.2 mL/min); temperature of injection port, 310 °C; temperature of flame-ionization detector, 320 °C; sample injection volume, 1 μL. The oven temperature were as follows: 210 °C for 1 min, raised at 8 °C/min to 215 °C, held for 1 min, raised at 20 °C/min to 350 °C, held for 1 min, raised at 0.5 °C/min to 355 °C, held 6 min. The peaks obtained in the chromatograph were characterized and quantified by injecting known amounts (0.63, 1.25, 2.5, and 5.0 μg/mL) of different synthesized triacylglycerols (POP, POS, POO, SOO and SOS) in 1 μL of trilaurin, which was used as an internal standard, to construct the calibration curve (POP, $r^2 = 0.992$; POS, $r^2 = 0.999$; POO, $r^2 = 0.996$; SOO, $r^2 = 0.983$; SOS, $r^2 = 0.989$).

The content (%, w/w) of each TG in analog or lard was determined by dividing the weight (mg) of TG in analog with the total weight (mg) of TGs in analog (Maehr, Zenchoff, & Coffen, 1994). Commercial refined, bleached and deodorized lard was analyzed to contain 19.0% POP, 31.9% POS, 42.8% POO, 1.9% SOS, and 4.4% SOO. Triplicate samples each were analyzed twice.

2.5. Yield of cocoa butter analog

Yield of cocoa butter analog prepared under optimized conditions (17 MPa, 50 °C, 3 h) with 0.06 mmol lard and 0.044 mmol tristearin (at a mole ratio 1.4) was calculated with the following equation:

$$\text{Yield of cocoa butter analog (\%)} = \text{Mole of cocoa butter analog} \times 100\%/\text{Mole of lard.}$$

Triplicate samples each were analyzed twice.

2.6. Lipase activity assay

Lipase hydrolytic activity was measured according to the method described by R'ua, Maurion, Fernande, Otero, and Ballesteros (1993) using *p*-nitrophenyl butyzate as substrate. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute.

2.7. Melting point determination

Melting point of cocoa butter analog was determined by the capillary tube method according to AOCS official method Cc1-25 (AOCS, 1989). Briefly, a capillary tube was dipped in the completely liquefied cocoa butter analog purified previously by a silica column until the sample rose to about 10 mm high in the tube. Then, the both ends of the tube were fused with a flame. After 16 h of refrigeration at 4 °C, the capillary tube was attached to the lower end of a mercury thermometer which was suspended in a 500 mL-beaker half-filled with distilled water in a water bath. Starting at 15 °C, heat was gently applied until the fat inside the tube was completely clear. Triplicate samples each were analyzed twice.

2.8. Water activity determination (a_w)

Water activity of reaction mixture was determined by a water activity meter (HygroPalm AW1 Set, Rotronic Instrument Corp., NY, USA) at 25 °C for 50 min.

2.9. Statistical analysis

Analysis of variance of results was carried out using General Linear Model Procedure of SAS Statistical Software, Version 6.11 (SAS, 1995). TG content versus lipase source, oil source, reaction time, reaction pressure, acyl donor, reaction temperature or lard/tristearin ratio and yield of cocoa butter analog were each tested in triplicates. Multiple comparisons of means were carried out by Duncan's multiple range test at $p < 0.05$.

3. Results and discussion

3.1. Optimization of cocoa butter analog formation in SC-CO_2 system

Interesterification of fat has been carried out with lipases, in either free or immobilized from, from various sources; however, due to the relative low stability of free lipases against pressure and temperature (Knez & Habulin, 2002), immobilized lipases have become the focus of recent researches and industrial application.

Four commercial free lipases (Lipases AP-6, G, PS, and *C. cylindracea* lipase Type VII) and one immobilized lipase (Lipozyme IM-20) were tested in a SC-CO_2 batch reactor at 10 MPa and 50 °C to compare their interesterification effect on cocoa butter analog production. As shown in Table 1, 1.2 U/g of immobilized *M. miehei* lipase, Lipozyme IM-20, showed remarkable catalysis and specificity for enzymatic synthesis of cocoa butter analog due to the most similar TG content (23.0% POP, 39.9% POS, 15.8% POO, 13.5% SOS, and 7.8% SOO) to the corresponding TG content (16.0% POP, 36.0% POS, 10.0% POO, 26.0% SOS, and 12.0% SOO) in nature cocoa butter. TG of analog prepared with 83.3 U/g of free lipase from *P. cepacia* also contained high (33.2%) level of POS; however, POO content was far higher than that (10%) in cocoa butter. Therefore, Lipozyme IM-20 was observed to efficiently catalyze interesterification of lard with tristearin and appeared to be suitable for the cocoa butter analog production. Different in lipase source such as from *Rhizopus arrhizus* might facilitate the interesterification reaction of palm oil with stearic acid or stearic acid ethyl ester in synthesizing CBEs (Bloomer, Adlercreutz, & Mattiasson, 1990; Mojovic, Siler-Marinkovic, Kukic, & Vunjak-Novakovic, 1993). However, the TG content of this product was less similar, than that prepared with Lipozyme IM-20 (Table 1), to that of cocoa butter.

Subsequently, oils from various sources were applied to the SC-CO_2 reactor and the TG contents of analogs obtained were compared (Table 2). Analog prepared with this refined lard apparently increased POS content to 39.9% and reduced POO content to 15.8%, comparing to the 35.1% POS and 20.3% POO content in product prepared with palm oil and tristearin (Table 2), superior to the results reported by Liu and Shaw (1997) using palm oil as oil source, due to the specific interesterification of lard and tristearin catalyzed by Lipozyme IM-20. It suggests that stearate in tristearin is effectively interesterified with oleate in POO to increase POS content and meanwhile, with oleate and palmitate in POO to increase SOS content in the presence of immobilized lipase. Therefore, it was clear that refined lard was favorable for the preparation of cocoa butter analog and was used as oil source in the following experiments. Similar results were also observed by Bloomer et al. (1990) that this 1,3-specific lipase efficiently converted POP in palm oil fraction into POS and SOS through interesterification reaction. However, reaction mixture using lard as oil source appears to suffer from a major drawback of cholesterol contamination (102 mg/100 g lard). Hence, an extra process of molecular distillation may be essential to remove cholesterol from cocoa butter analog posterior to silica column separation, as suggested by Lanzanl et al. (1994).

Reaction time was set from 1 to 7 h to evaluate the time effect on the TG content of analog at 10 MPa and 50 °C

Table 1
Triacylglycerol content of cocoa butter analog synthesized by lipases from various sources[A]

Lipase source	Trade name or brand	Enzyme activity (units/g)[B]	Triacylglycerol content (w/w, %)				
			POP	POS	POO	SOS	SOO
Aspergillus niger	Amano AP-6	81.8	17.0 ± 1.8^{a}	29.2 ± 1.9^{a}	45.4 ± 2.0^{d}	4.2 ± 0.5^{a}	4.2 ± 0.6^{a}
Candida cylindracea type VII	Sigma	68.7	21.9 ± 1.5^{b}	29.1 ± 1.7^{a}	31.4 ± 2.4^{c}	8.4 ± 1.0^{b}	9.2 ± 0.8^{c}
Mucor miehei	Lipozyme IM-20	1.20	23.0 ± 0.4^{bc}	39.9 ± 1.2^{c}	15.8 ± 0.8^{a}	13.5 ± 0.5^{c}	7.8 ± 0.7^{b}
Penicillium camembertii	Amano G	43.1	18.8 ± 0.3^{ab}	31.2 ± 0.3^{b}	20.9 ± 0.1^{b}	14.4 ± 0.0^{c}	14.7 ± 0.4^{d}
Pseudomonas cepacia	Amano PS	83.3	22.6 ± 1.6^{bc}	33.2 ± 1.4^{b}	22.8 ± 1.3^{b}	13.3 ± 0.7^{c}	8.1 ± 0.4^{b}

P, palmitate; O, oleate; S, stearate.

Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).

[A] Each lipase (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard, 0.02 mmol tristearin and 5 μL of water in SC-CO_2 at 10 MPa and 50 °C for 3 h.

[B] Enzyme activity assay was conducted by hydrolyzing *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol per min.

Table 2
Effect of oil source on triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20[A]

Oil source	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
Original oil					
Palm oil	43.4 ± 0.5	6.2 ± 0.3	48.5 ± 0.3	0.0 ± 0.0	1.9 ± 0.1
Lard	19.0 ± 1.0	31.9 ± 0.6	42.8 ± 2.1	1.9 ± 1.1	4.4 ± 1.0
Beef tallow	26.1 ± 0.6	22.6 ± 0.5	40.9 ± 0.2	4.0 ± 0.2	6.4 ± 0.3
After interesterification reaction					
Palm oil	21.6 ± 0.2^{b}	35.1 ± 0.1^{b}	20.3 ± 0.0^{bc}	14.1 ± 0.2^{a}	8.9 ± 0.4^{ab}
Lard	23.0 ± 0.4^{bc}	39.9 ± 1.2^{c}	15.8 ± 0.8^{a}	13.5 ± 0.5^{a}	7.8 ± 0.7^{a}
Beef tallow	19.3 ± 0.3^{a}	32.7 ± 0.8^{a}	18.8 ± 1.2^{b}	17.4 ± 0.9^{b}	11.8 ± 0.7^{c}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol oil, 0.02 mmol tristearin and 5 μL of water in SC-CO_2 at 10 MPa and 50 °C for 3 h.

using lard and tristearin as substrates (Table 3). Apparently, the content of POS, the major component in cocoa butter, increased rapidly from 31.9% in lard to 36.9% at 1 h but slowly to 39.9% at 3 h. Similar trend in the change of SOS level was observed. Meanwhile, POO level decreased sharply from 42.8% in lard to 18.3% at 1 h and then slowly to 15.8% at 3 h. Comparing to the TG content of analog at 3 h, content of the corresponding TG changed slightly after 7-h interesterification reaction. Therefore, on the basis of the above findings in Table 3, reaction time of 3 h appeared to be optimal and was used in the following experiments.

In biocatalytic processes, the operating conditions are preferably restricted to preserve the enzyme stability and to be practical in processing. Accordingly, optimization of pressure versus TG content was conducted. Though no apparent difference among the TG contents in three products was observed, TG composition of analog prepared at 17 and 24 MPa appeared to be much similar to that of natural cocoa butter (Table 4). In addition, no reduction in enzyme activity was detected after experiment under 17 MPa (data not shown). In a preliminary test, Lipozyme IM-20 retained more than 50% of enzyme activity after a 34-h interesterification in SC-CO_2 at 17 MPa and 50 °C for the synthesis of cocoa butter analog, while marked reduction in enzyme activity was observed when reaction was conducted at higher pressure (data not shown). Therefore, pressure of 17 MPa was applied in the following experiments.

Knez and Habulin (2002) studied the changes in activity of some lipases and esterases upon contacting with carbon dioxide and found that pressure treatment unapparently affected the enzyme performance. On the contrary, Erickson, Schyns, and Cooney (1990) and Kim et al. (1994) reported that the rates of acidolysis and esterification decreased with the increasing reaction pressure at a constant temperature in SC-CO_2 or supercritical ethane. The reason for this consequence could be partly due to the changes in solubility of substrates and in partition of reactants between the supercritical solvent phase and the immobilized enzyme phase and partly due to the intrinsic properties of enzyme (Erickson et al., 1990). The increase in pressure of a SC-CO_2 reactor results in a decrease in diffusion as the results of the increase in the density and viscosity of liquid CO_2.

Source of acyl donor plays an important role in interesterification reaction of oils. Among the acyl donors used, stearic acid, stearic acid ethyl ester, and stearic anhydrate appeared to be poor acyl donors for the interesterification reaction with lard (Table 5) since the POP content (32–35%) and POO content (18–20%) in products was far higher than the corresponding TG content in cocoa butter and analog prepared with tristearin. Apparently, tristearin was a suitable acyl donor for the interesterification reaction with lard catalyzed by Lipozyme IM-20. Similar results were also observed by Liu and Shaw (1997) in preparing cocoa butter analog in a SC-CO_2reactor, while stearic acid and stearic acid ethyl ester were not acceptable as

Table 3
Triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20 in SC-CO_2 as affected by the reaction period of time[A]

Reaction time (h)	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
1	23.8 ± 0.7^{a}	36.9 ± 1.4^{a}	18.3 ± 1.2^{b}	12.3 ± 1.1^{a}	8.7 ± 1.0^{ab}
3	23.0 ± 0.4^{a}	39.9 ± 1.2^{b}	15.8 ± 0.8^{a}	13.5 ± 0.5^{ab}	7.8 ± 0.7^{a}
7	24.0 ± 1.1^{ab}	38.4 ± 1.9^{ab}	15.6 ± 1.2^{a}	14.0 ± 1.8^{b}	8.0 ± 1.9^{a}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard, 0.02 mmol tristearin and 5 μL of water in SC-CO_2 at 10 MPa and 50 °C for various reaction periods of time.

Table 4
Triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20 in SC-CO_2 as affected by reaction pressure[A]

Reaction pressure (MPa)	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
10	23.0 ± 0.4^{ab}	39.9 ± 1.2^{ab}	15.8 ± 0.8^{b}	13.5 ± 0.5^{a}	7.8 ± 0.7^{a}
17	22.6 ± 0.4^{a}	38.5 ± 0.9^{a}	14.9 ± 0.2^{b}	15.1 ± 0.4^{b}	8.9 ± 1.3^{ab}
24	22.3 ± 2.0^{a}	39.7 ± 2.1^{ab}	12.9 ± 0.8^{a}	16.6 ± 2.2^{b}	8.5 ± 1.2^{ab}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard, 0.02 mmol tristearin and 5 μL of water in SC-CO_2 at various pressures and 50 °C for 3 h.

Table 5
Effect of acyl donors on triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20[A]

Acyl donor source	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
Stearic acid	34.5 ± 1.6^{b}	34.5 ± 1.2^{a}	20.0 ± 1.2^{bc}	6.3 ± 0.9^{a}	4.7 ± 0.7^{a}
Stearic acid ethyl ester	34.8 ± 1.3^{b}	34.9 ± 1.3^{a}	18.3 ± 1.2^{b}	7.4 ± 0.7^{ab}	4.6 ± 1.0^{a}
Stearic anhydride	32.0 ± 1.3^{b}	34.8 ± 1.0^{a}	18.1 ± 1.5^{b}	7.7 ± 0.7^{ab}	7.4 ± 1.1^{b}
Tristearin	22.6 ± 0.4^{a}	38.5 ± 0.9^{b}	14.9 ± 0.2^{a}	15.1 ± 0.4^{c}	8.9 ± 1.3^{bc}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard, 0.02 mmol acyl donor and 5 μL of water in SC-CO_2 at 17 MPa and 50 °C for 3 h.

acyl-acceptors. Different lipase such as that from *R. arrhizus* was indicated to facilitate the interesterification reaction of palm oil with stearic acid or stearic acid ethyl ester in CBE preparations (Bloomer et al., 1990; Mojovic et al., 1993).

Lipozyme IM-20 is an immobilized lipase and is relatively stable in a SC-CO_2 system at temperatures lower than 80 °C (Chulalaksananukul, Condoret, & Combes, 1993). However, slight change in reaction temperature may affect the interesterification reaction rate of oils and results in the change of TG content of analog. In Table 6, it was clear that POS content increased rapidly from 31.9% in lard to 36.0% at 40 °C and then slowly to 38.5% in products at 50 and 60 °C (Table 6). On the contrary, POO content decreased sharply from 42.8% in lard to 19.4% at 40 °C and then to about 15% in products at 50 and 60 °C. It suggests that reaction at temperature higher than 50 °C is insignificantly ($p > 0.05$) favorable for the interesterification of lard and tristearin catalyzed by this immobilized lipase. Reaction temperature affects strongly the partition of substrates between the SC-CO_2 phase and enzyme phase (Shishikura, Fujimoto, Suzuki, & Arai, 1994) as the results of the changes in substrate solubility and the density and viscosity of SC-CO_2 (Chrastill, 1982). Optimal diffusion is beneficial for mass transfer between substrates and products. Therefore, on the basis of operation cost, optimal temperature was determined to be 50 °C.

To optimize the effect of lard/tristearin mole ratio on TG content of analog, various ratios of lard/tristearin (8.6–1.0, mol/mol) were applied to the reactor and the interesterification reaction was carried out at 17 MPa and 50 °C for 3 h (Table 7). Apparently, higher in lard levels decreased the POS and SOS contents and increased the POP and POO contents in analog (Table 7). POS content in analog reached 38.5%, similar to that (about 36%) in cocoa butter, when the ratio was 2.9, while suffering from a higher POO content and POP content and a lower

Table 6
Triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20 in SC-CO_2 as affected by reaction temperature[A]

Reaction temperature (°C)	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
40	26.3 ± 0.5^{c}	36.0 ± 1.0^{a}	19.4 ± 0.1^{b}	11.3 ± 0.5^{a}	7.0 ± 1.0^{c}
50	22.6 ± 0.4^{a}	38.5 ± 0.9^{b}	14.9 ± 0.2^{a}	15.1 ± 0.4^{bc}	8.9 ± 1.3^{b}
60	23.7 ± 1.6^{ab}	38.5 ± 1.0^{b}	14.7 ± 0.5^{a}	14.7 ± 0.4^{b}	8.4 ± 0.8^{b}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard, 0.02 mmol tristearin and 5 μL of water in SC-CO_2 at 17 MPa, various temperatures for 3 h.

Table 7
Effect of lard/tristearin mole ratio on triacylglycerol content of cocoa butter analog synthesized by a lipase of lipozyme IM-20 in SC-CO_2[A]

Lard/triesterate[B]	Triacylglycerol content (w/w, %)				
	POP	POS	POO	SOS	SOO
8.6	27.3 ± 0.4^{d}	32.3 ± 1.3^{a}	25.9 ± 1.4^{e}	6.6 ± 0.3^{a}	7.9 ± 0.3^{a}
5.7	26.3 ± 0.6^{d}	35.5 ± 0.6^{b}	21.2 ± 0.9^{d}	9.0 ± 0.6^{b}	8.0 ± 0.6^{a}
2.9	22.6 ± 0.4^{c}	38.5 ± 0.9^{c}	14.9 ± 0.2^{c}	15.1 ± 0.4^{c}	8.9 ± 0.3^{b}
1.4	17.8 ± 0.3^{b}	40.8 ± 1.0^{d}	10.2 ± 0.3^{b}	23.2 ± 0.8^{d}	8.0 ± 0.2^{a}
1.0	13.7 ± 0.6^{a}	41.0 ± 1.1^{d}	7.3 ± 0.4^{a}	30.2 ± 1.5^{e}	7.8 ± 0.5^{a}

P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations. Mean values in the same column with a letter in common do not differ significantly ($p > 0.05$).
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of various mole ratios of lard/tristearin and 5 μL of water in SC-CO_2 at 17 MPa, 50 °C for 3 h.
[B] Mole ratio of lard to tristearin.

SOS content than the corresponding TG in cocoa butter. Decreased in ratio to 1.0 declined the POP content to 13.7% and POO to 7.3%, while increasing SOS content to 30.2%, and thus, apparently unfavorable for the production of cocoa butter analog due to the greater difference in TG content from natural product. Therefore, mole ratio of lard/tristearin for analog production was optimized to be 1.4. High level of lard as a substrate might inhibit the catalysis of lipase during the interesterification reaction (Marty, Chulalaksananukul, Condoret, Willemot, & Durand, 1990), and importantly, decreased the production of analog as a result of the increasingly

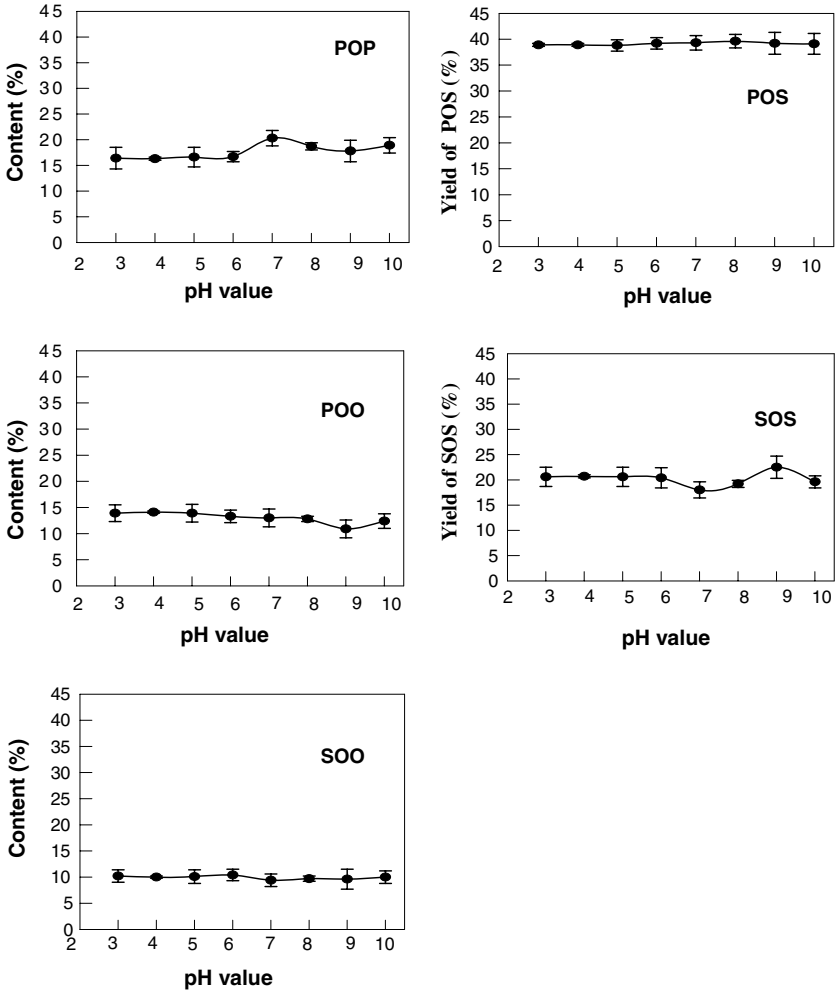

Fig. 1. Changes in TG content of cocoa butter analog versus pH value. Lipozyme IM-20 in 10 mM mixed Good's buffer solution with various pH values was lyophilized prior to reaction with a 40 mL reaction mixture of 0.06 mmol lard and 0.02 mmol tristearin in SC-CO_2 at 17 MPa, 50 °C for 3 h. Each value is the mean of triplicate determinations.

excessive intact substrate. Besides, purification cost by a subsequent Silica column would be increasing.

Water availability in a reaction system obviously affects the enzyme activity since water may serves not only as a solvent but also a reactant in hydrolytic reactions. Therefore, water activity (a_w) is considered as an important thermodynamic parameter that represents the enzyme hydration level in a non-aqueous medium, such as SC-CO_2 system, and modulates the equilibrium between hydrolytic and synthetic processes (Dudal & Lortie, 1995; Svensson, Wehtje, Adlercreutz, & Mattiasson, 1994). In the present study, the change in TG in analog versus water content (0–25%; a_w, 0.61–0.91) in a SC-CO_2 system was studied at 17 MPa and 25 °C for 3 h; however, only slight changes (<5%) in POP and SOS contents were observed (data not shown). Hence, for convenience, cocoa butter analog was produced under the anhydrous system (a_w, 0.61). Condoret, Vankan, Joullia, and Marty (1997) indicated that the optimum water content for Lipozyme IM-20 was between 8% and 12% (g/g of supported enzyme) (a_w, 0.4–0.7). The difference in water content required could be due to the difference in substrates used and reaction conditions. Presence of water in reaction system changes/affects the solubilities of substrates and products as well as mass transfer resistance.

Lipase in buffer solutions with various pH values (pH 3–10) was lyophilized to conduct the interesterification reaction in SC-CO_2. TG content of cocoa butter analog was not apparently affected by acidic pH values (pH < 6); however, POP increased by about 4% and SOS decreased by about 3% at pH 7 (Fig. 1), apparently unfavorable for the preparation of cocoa butter analog. POS, the main component in cocoa butter, and SOO retained at the same level of about 38% and 10%, respectively, at all pH values; however, SOS content increased to about 23% and POO content decreased to 11% at pH 9, closing to the corresponding TG content in cocoa butter. Hence, optimal pH value for interesterification reaction of lard with tristearin by Lipozyme IM-20 was suggested to be at pH 9.

Lipase, previously lyophilized at various pH values in aqueous solution, usually conducts the interesterification reaction in anhydrous organic solvents with the same enzyme activity as it does at the corresponding pH value in aqueous solution. Such behavior of lipase is termed as pH memory (Klibanov, 1989). Variation in pH value may cause the changes in enzyme conformation, solubility of substrate and the interactions of substrates with enzyme, and thus, to the changes in TG contents of cocoa butter analog (Fig. 1) (Goh, Yeong, & Wang, 1994).

3.2. Yield of cocoa butter analog

Yield of cocoa butter analog prepared under optimized conditions (17 MPa, 50 °C, 3 h) with 0.06 mmol lard and 0.044 mmol tristearin was compared with that prepared with 0.06 mmol palm oil and 0.03 mmol tristearin (Liu & Shaw, 1997) and the results are shown in Table 8. It was clear that yield of the former analog was 63%, higher than that (53%) of latter analog, while the melting points of both products were very similar (34.5 and 34.3 °C, respectively). It reveals that lard is an effective substrate in increasing the yield.

Table 8
Yield (%) of cocoa butter analog by interesterification of lard or palm oil with tristearin in SC-CO_2 with a lipase of lipozyme IM-20[A]

Oil	Yield (%)	m.p.[B] (°C)
Lard	63.0 ± 0.3	34.5 ± 0.7
Palm oil	53.0 ± 0.5	34.3 ± 0.6

Yield of cocoa butter analog (%) = Mole of cocoa butter analog × 100%/Mole of lard.
P, palmitate; O, oleate; S, stearate.
Each value is the mean of triplicate determinations.
[A] Lipozyme IM-20 (90 mg) was added to a 40 mL reaction mixture of 0.06 mmol lard–0.044 mmol tristearin or 0.06 mmol lard–0.03 mmol tristearin in SC-CO_2 at 17 MPa, 50 °C for 3 h to prepare analog.
[B] Melting point.

In conclusion, interesterification reaction conditions were optimized to be at 17 MPa and 50 °C for 3 h using lard and tristearin, at a mole ratio of 1.4, as substrates in a SC-CO_2 system with a yield of about 63%. However, an additional process of molecular distillation may be required to minimize cholesterol content in obtained cocoa butter analog posterior to a Silica column purification.

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