Synthesis of Cocoa Butter Equivalent by Lipase-Catalyzed Interesterification in Supercritical Carbon Dioxide

Kuan-Ju Liu[a], Hong-Ming Cheng[b], Rey-Chang Chang[c], and Jei-Fu Shaw[a,*]

[a]Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529, [b]Department of Marine Food Science, National Taiwan Ocean University, Keelung, Taiwan 20224, and [c]Department of Sea-Food Technology, China Junior College of Marine Technology, Taipei, Taiwan 111

ABSTRACT: With supercritical carbon dioxide as a reaction medium, the syntheses of cocoa butter equivalent by interesterification with various lipases were investigated. The study showed that among those five lipases tested, lipase IM-20 from *Mucor miehei* was the most effective and specific in synthesizing this cocoa butter equivalent product by interesterification. The yields of cocoa butter equivalent are affected by pressure, substrate oil composition, solubility and co-solvent. The best reaction conditions were: reaction pressure at 1500 psi, triglyceride with high content of POP (P, palmitate; O, oleate) and POO, reaction medium with 5.0% water, and reaction temperature at 50°C. The major component of cocoa butter, POS (S, stearate), can be increased by 6.0% by adding a small amount of carbon dioxide. The yield and melting point of the purified cocoa butter equivalent are 53.0% and 34.3°C, respectively.
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KEY WORDS: Cocoa butter equivalent, interesterification, lipase, palm oil, supercritical carbon dioxide.

Cocoa butter, which amounts to 25–36% in finished chocolate, is responsible for the smooth texture, contractibility, flavor release, and gloss of the product (1). Much attention has been given to the production of cocoa butter equivalent from fats and oils of lower value *via* interesterification with lipase in organic solvent (2–5), but enzymatic synthesis of cocoa butter equivalent in supercritical media is still an unexplored area of research. Supercritical fluids can be used as solvents for enzymatic reactions (6–9). Supercritical carbon dioxide (SC-CO_2) can be useful as an excellent medium for interesterification and transesterification. Nakamura *et al.* (10) have presented the first study of lipase-catalyzed triglyceride interesterification, and Chi *et al.* (11) have pointed out the influence of water. Pasta *et al.* (8) described a subtilisin-catalyzed transesterification between *N*-acetyl-L-phenylalanine chloroethyl ester and ethanol in supercritical fluid. The attractiveness of CO_2 as a process solvent comes from its low toxicity, negligible environmental impact, and low cost (CO_2 is the most inexpensive commercially available organic solvent) (7,12).

*To whom correspondence should be addressed at Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529.
E-mail: boplshaw@ccvax.sinica.edu.tw.

In the present work, we found that cocoa butter equivalent could be efficiently synthesized by interesterification with lipase from *Mucor miehei* (lipozyme IM-20) as biocatalyst in SC-CO_2. The effect of temperature, pressure, lipase, acyl donors, water content, addition of cosolvent, type of oil, metal salts, and pH memory is also described.

EXPERIMENTAL PROCEDURES

Materials. Lipases from *Aspergillus niger, Penicillium camembertii* (Amano "G"), and *Pseudomonas cepacia* (Amano "PS") were purchased from Amano International Enzyme Co. (Nagoya, Japan). Lipase from *Candida cylindracea* Type VII was purchased from Sigma Chemical Co. (St. Louis, MO). Immobilized lipase IM-20 from *M. miehei* was supplied by Novo Nordisk Inc. (Danbury, CT). Hexane, *t*-butyl alcohol, $CaCl_2$, $CuCl_2$, $SrCl_2$, $ZnCl_2$, and ethanol were obtained from Merck Chemical Co. (Darmstadt, Germany). Olive oil, stearic acid, stearic acid ethyl ester, stearic anhydride, and tristearin were purchased from Sigma Chemical Co. POP, POO, POS, SOS, and SOO (P, palmitate; O, oleate; S, stearate were purchased from Matreya, Inc. (Pleasant Gap, PA). Palm oil was purchased from First Chemical Co. (Taipei, Taiwan). Peanut oil was purchased from Chang Tai Co. (Taipei, Taiwan). CO_2 was purchased from Qing Feng Co. (Taipei, Taiwan).

Methods. The experimental apparatus with SC-CO_2 as the reaction medium is schematically shown in Figure 1. The reactor was a 40-mL high-pressure vessel, into which 0.09 g immobilized lipase, 60 μL palm oil, 0.03 g tristearin, 5 μL water, and 70 μL mixed solvent of *n*-hexane and *t*-butyl alcohol (9:1, vol/vol) were loaded. The reaction was incubated at 50°C for 3 h. At various time intervals, 0.5 μL of the reaction mixture was withdrawn and analyzed by gas chromatography (Hitachi model G-3000; Hitachi, Tokyo, Japan). An Rtx®-65TG fused-silica capillary column of 30 m × 0.25 mm i.d. (Restek Corporation, Bellefonte, PA) was used. Hydrogen gas was chosen as the carrier gas at a flow rate of 1.2 mL/min. The injection port and flame-ionization detector temperatures were 310°C and 320°C, respectively. The temperature program was: 210°C (8°C/min) to 215°C (20°C/min) to 350°C,

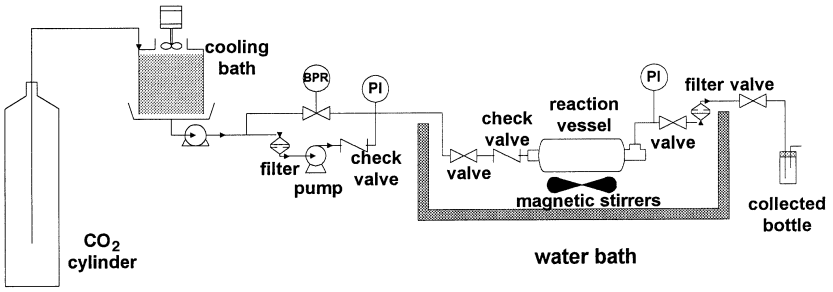

FIG. 1. Schematic diagram of supercritical carbon dioxide system for enzymatic reaction. Abbreviations: BPR, back pressure regulator; PI, pressure indicator.

holding 1 min, (0.5°C/min) to 355°C, and holding 6 min. The product compositions were quantitated by an integrator with trilaurin as internal standard.

To study the effect of water content on the lipase-catalyzed synthesis, the enzyme was lyophilized with a Savant Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY) at 50 millitorr for 24 h. Water was removed from organic media by 3 Å molecular sieves (Merck). To study the pH effect on synthesis, the lipase was dissolved in 10 mM mixed Good's butter solution of different pH {10 mM each of *N*,*N*-bis(2-hydroxyethyl)-glycin, 3-(cyclohexylamino)-1-propanesulfonic acid, sodium acetate, and 1,3-bis[tris(hydroxymethyl)-methylamino]propane were mixed and adjusted to various pH values by either concentrated HCl or NaOH} and then lyophilized as described earlier.

Measurement of lipase. The lipase hydrolytic activities were measured according to the method described by R'ua *et al.* (13).

RESULTS AND DISCUSSION

Among five commercial lipases tested (Lipases AP-6, G, PS, *C. cylindracea* lipase Type VII, and IM-20), *M. miehei* lipase IM-20 showed the best catalytic efficiency and specificity for enzymatic synthesis of cocoa butter equivalent (Table 1). The lipozyme IM-20-catalyzed interesterification of palm oil with tristearin was efficient. The yields of POP, POS, POO, SOS, and SOO in the purified oil were 18.0, 36.3, 19.0, 13.4, and 13.3%, respectively, from 60 µL palm oil when reacted with 0.03 mmol tristearin, catalyzed by 90 mg lipase in 40 mL supercritical carbon dioxide at 1500 psi, 50°C (Table 2). The yields of POP, POS, POO, SOS, and SOO were affected by type of oil, acyl donor, reaction temperature, reaction pressure, cosolvents, water content, metal salts, and pH memory.

As shown in Table 2, the best compositions of cocoa butter equivalent were produced by the reaction of palm oil and tristearin. The compositions were not satisfactory when the reaction was carried out with other oils. This could be due to the effects of different compositions. Therefore, it is desirable to carry out the interesterification reaction with palm oil.

The effects of reaction time for the lipase-catalyzed interesterification are shown in Table 3. Good compositions increased as the reaction time was increased up to 3 h. Stearic acid ethyl ester appeared to be a poor substrate for lipase-catalyzed synthesis of cocoa butter equivalent (Table 4). Tristearin appeared to be the best acyl donor for the lipase-catalyzed synthesis of cocoa butter equivalent. As shown in Table 4, the yields of POP, POS, POO, SOS, and SOO were 20.5, 37.7, 16.9, 14.3, and 10.6%, respectively, with 0.03 mmol triacylglycerol as acyl donor.

The temperature effect is shown in Table 5. The yields of POS and SOS increased slightly as the temperature was in-

TABLE 1
Triglyceride Formation by Lipase from Different Sources[a]

Source	Trade name or brand	Triglyceride species[b] (%)					Hydrolysis[c] act. (unit/g)
		POP	POS	POO	SOS	SOO	
Aspergillus niger	Amano AP-6	40.4	12.5	40.9	2.5	3.7	908.6
Candida cylindracea type VII	Sigma	42.3	17.6	31.2	4.0	4.9	762.8
Mucor miehei	Novo IM20	31.8	24.2	30.2	4.3	9.5	13.6
Penicillium camembertii	Amano G	36.7	24.1	29.3	2.8	7.1	479.1
Pseudomonas cepacia	Amano PS	32.8	23.5	30.2	5.0	8.5	925.9

[a]The lipase (0.05 g) was added to a reaction mixture (40 mL) containing 100 µL palm oil, 0.014 mmol tristearin, 45 µL hexane, 5 µL *t*-butyl alcohol, and 5 µL water in 3500 psi supercritical carbon dioxide at 50°C for 15 h. Amano AP-6, G, and PS (Amano International Enzyme Co., Nagoya, Japan); Sigma (St. Louis, MO); Novo IM20 (Novo Nordisk Inc., Danbury, CT).

[b]P = palmitate; O = oleate; S = stearate.

[c]Hydrolysis activity was measured by the hydrolysis of *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme that produced 1 µmol of *p*-nitrophenol per minute.

[d]Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.

TABLE 2
Effect of Different Oils on the Lipase-Catalyzed Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
Oils	POP	POS	POO	SOS	SOO
Native oils					
Palm oil	43.4	6.2	48.5	0.0	1.9
Olive oil	13.5	2.6	77.0	0.0	6.9
Peanut oil	24.5	9.7	54.5	0.0	11.3
Interesterified oils					
Palm oil	21.6	38.7	16.7	14.1	8.9
Olive oil	4.0	23.0	18.0	25.5	29.5
Peanut oil	4.7	24.9	34.4	25.5	10.4
Purified oil					
Palm oil	18.0	36.3	19.0	13.4	13.3

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing various oils (60 µL), 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 1500 psi supercritical carbon dioxide at 50°C for 3 h.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 3
Kinetics of Lipase-Catalyzed Transesterification of Palm Oil with Tristearin at Various Times[a]

Reaction time	Formation of triglycerides[b] (%)				
(h)	POP	POS	POO	SOS	SOO
1	24.2	28.1	16.5	17.7	13.5
3	20.5	36.6	16.9	14.2	11.8
7	20.5	37.7	16.9	14.3	10.6

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 3500 psi supercritical carbon dioxide at 50°C. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 4
Effect of Acyl Donors on the Lipase-Catalyzed Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
Acyl donor	POP	POS	POO	SOS	SOO
Stearic acid	32.9	22.5	32.3	3.9	8.4
Stearic acid ethyl ester	32.5	20.3	37.4	2.5	7.3
Stearic anhydride	25.9	30.4	26.9	7.1	9.7
Tristearin	20.5	37.7	16.9	14.3	10.6

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 µL palm oil, various acyl donors (0.03 mmol), 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 1500 psi supercritical carbon dioxide at 50°C for 7 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 5
Kinetics of Lipase-Catalyzed Transesterification of Palm Oil with Tristearin at Various Temperatures[a]

Reaction temperature	Formation of triglycerides[b] (%)				
(°C)	POP	POS	POO	SOS	SOO
40	26.7	33.0	22.9	8.3	9.1
50	20.5	36.6	16.9	14.2	11.8
60	20.6	37.0	17.3	14.3	10.8

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 3500 psi supercritical carbon dioxide at various temperatures for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

creased to 50°C. There is no significant effect on the activity of lipase in SC-CO_2 when pressure is increased from 1500 psi pressure to 3500 psi (Table 6). The optimal pressure was 1500 psi. The effects of the palm oil/tristearin ratio in the reaction mixture on the lipase-catalyzed interesterification are shown in Table 7. The optimal composition of cocoa butter equivalent increased as the ratio of palm oil/tristearin was decreased to 60:0.06 (μL/g). Higher palm oil levels decreased the yield of cocoa butter equivalent.

Table 8 shows the effect of water content on the synthesis of cocoa butter equivalent. Lipase IM-20 performs best at a water content of 5% (vol/vol) for the interesterification of palm oil with tristearin. A small amount of water is needed to maintain enzyme activity. However, at higher water content, the degree of synthesis gradually declined. Table 9 compares the yields of cocoa butter equivalent at 50°C for 3 h for SC-CO_2 and hexane. The rate of interesterification is significantly greater and faster in SC-CO_2 than in hexane. We suggest that mass transport from the bulk solution to the enzyme is faster in SC-CO_2 than in *n*-hexane.

Addition of cosolvent affected the yield of cocoa butter equivalent. Table 10 shows the effect of mixed solvent of hexane and ethanol on the synthesis of cocoa butter equivalent. It appears that the yield of cocoa butter equivalent was much better in mixed solvent (SC-CO_2/hexane/*t*-butyl alcohol = 5714:9:1) than in pure SC-CO_2. The effect of cosolvents

TABLE 6
Kinetics of Lipase-Catalyzed Transesterification of Palm Oil with Tristearin at Various Pressures[a]

Reaction pressure	Formation of triglycerides[b] (%)				
(psi)	POP	POS	POO	SOS	SOO
1500	21.6	38.7	16.7	14.1	8.9
2500	20.4	35.9	17.7	14.5	11.5
3500	20.5	36.6	16.9	14.2	11.8

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 μL palm oil, 0.03 mmol tristearin, 63 μL hexane, 7 μL *t*-butyl alcohol, and 5 μL water in supercritical carbon dioxide at 50°C and various pressures for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 7
Effect of Palm Oil/Tristearin Ratio on the Synthesis of Cocoa Butter Equivalents[a]

Ratio (palm oil	Formation of triglycerides[b] (%)				
μL/tristearin g)	POP	POS	POO	SOS	SOO
180:0.03	34.2	27.7	29.5	3.3	5.3
120:0.03	29.8	30.2	26.5	5.6	7.9
60:0.03	21.6	38.7	16.7	14.1	8.9
60:0.06	13.2	36.0	7.4	32.1	11.2
60:0.09	12.0	35.7	4.8	38.2	9.3

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing various ratios of palm oil/tristearin, 63 μL hexane, 7 μL *t*-butyl alcohol, and 5 μL water in 1500 psi supercritical carbon dioxide at 50°C for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 8
Effect of Water on the Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
Added water (%)	POP	POS	POO	SOS	SOO
0	23.4	35.9	18.8	12.9	9.0
5	21.6	38.7	16.7	14.1	8.9
10	26.3	35.0	17.2	12.9	8.6
15	22.2	33.6	19.4	13.6	11.2
20	20.9	34.8	18.7	13.4	12.2
25	24.6	32.0	20.0	11.5	11.9

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 μL palm oil, 0.03 mmol tristearin, 63 μL hexane, 7 μL *t*-butyl alcohol in 1500 psi supercritical carbon dioxide with various amounts of added water at 50°C for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 9
Effect of Reaction System on the Lipase-Catalyzed Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
Reaction system	POP	POS	POO	SOS	SOO
SC-CO_2	21.6	38.7	16.7	14.1	8.9
Hexane	27.2	29.8	23.4	10.4	9.2

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 1500 psi supercritical carbon dioxide and hexane at 50°C for 3 h, respectively. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 10
Effect of Cosolvent on the Lipase-Catalyzed Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
System	POP	POS	POO	SOS	SOO
SC-CO_2	23.6	35.8	20.2	11.1	9.3
SC-CO_2/hexane/ethanol (40:0.063:0.007, vol/vol/vol)	27.3	36.6	18.9	9.0	8.2
SC-CO_2/hexane/*t*-butyl alcohol (40:0.063:0.007, vol/vol/vol)	21.6	38.7	16.7	14.1	8.9

[a]The lipase (0.09 g) was added to a reaction mixture (40 mL) containing 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL various cosolvents, and 5 µL water in 1500 psi supercritical carbon dioxide at 50°C for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

TABLE 11
Effect of Palm Oil/Tristearin Ratio on the Synthesis of Cocoa Butter Equivalents[a]

	Formation of triglycerides[b] (%)				
Metal salts	POP	POS	POO	SOS	SOO
None	21.6	38.7	16.7	14.1	8.9
$CaCl_2$	16.3	36.0	13.9	21.5	12.3
$CuCl_2$	22.4	33.5	19.1	13.1	11.9
$MgCl_2$	23.0	33.2	19.2	14.6	10.0
$MnCl_2$	21.0	35.4	17.6	13.9	12.1
$SrCl_2$	15.5	37.4	12.8	22.9	11.4
$ZnCl_2$	27.3	31.2	22.3	10.3	8.9

[a]Various metal salts (1 mM) and lipase (0.09 g) were added to a reaction mixture (40 mL) containing 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 1500 psi supercritical carbon dioxide at 50°C for 3 h. Palm oil composition: POP: 43.4%; POS: 6.2%; POO: 48.5%; SOS: 0%; SOO: 1.9%.
[b]P = palmitate; O = oleate; S = stearate.

could be due to a change in enzyme specificity. We also have studied the effect of metal ions on the rate of enzyme reaction by adding different metal ions in the reaction media. No significant variation in the reaction rates has been observed, but the addition of $CaCl_2$ and $SrCl_2$ can change the specificity of the lipozyme IM-20 in SC-CO_2 (Table 11).

The yield of cocoa butter equivalent was also affected by the pH of the aqueous solution from which the lipase was lyophilized, a phenomenon called "pH memory" by Klibanov (14). As shown in Figure 2, the optimal pH was 7 for the lyophilized lipase-catalyzed synthesis of cocoa butter equivalent. This possibly reflected the fact that changes in enzyme conformation, resulting from pH changes, affected the interesterification reaction of palm oil with tristearin. The synthesized cocoa butter equivalent had a 53.0% yield and a melting point of 34.3°C after the removal of fatty acid and temperature fractionation (Fig. 3).

The stability of the enzyme in SC-CO_2 was affected by the different sources of enzyme, type of used oil, reaction time, acyl donor, temperature, pressure, palm oil/tristearin ratio, water content, cosolvent, metal salt, and pH memory. The rate of enzyme reaction in SC-CO_2 is faster than in an organic solvent.

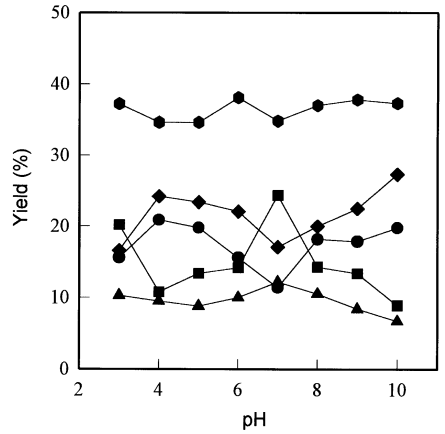

FIG. 2. Dependence of cocoa butter equivalent formation on pH of the aqueous solution from which the lipase was lyophilized. Experimental conditions: 0.09 g lipase was added to a reaction mixture (40 mL) that contained 60 µL palm oil, 0.03 mmol tristearin, 63 µL hexane, 7 µL *t*-butyl alcohol, and 5 µL water in 1500 psi supercritical carbon dioxide at 50°C for 3 h; (diamond) POP, (hexagon) POS, (circle) POO, (square) SOS, (triangle) SOO (S, stearate; O, oleate; P, palmitate).

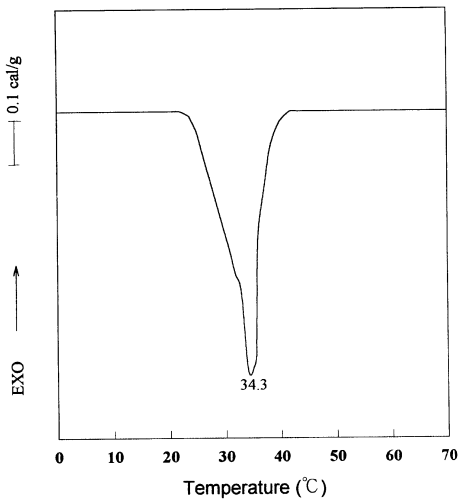

FIG. 3. Differential scanning calorimetry thermograms of cocoa butter equivalents.

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