Transesterification of Cocoa Butter by Fungal Lipases: Effect of Solvent on 1,3-Specificity

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Transesterification and alcoholysis reactions catalyzed by immobilized lipases from *Mucor miehei* and *Humicola lanuginosa* in hexane gave fatty acid esters that did not reflect the expected 1,3-specificity of the enzymes, due to competing acyl migrations in the partial glyceride products. However, both lipases were 1,3-specific in reactions when diethyl ether was used as a solvent, and this provided a convenient analytical methodology in combination with gas chromatography and nuclear magnetic resonance spectroscopy for the determination of fatty acid distribution within triglycerides.

KEY WORDS: Acyl migration, cocoa butter, *Humicola lanuginosa*, *Mucor miehei*, solvent effect, 1,3-specific lipase, transesterification.

In recent years, the use of lipases as biocatalysts to produce useful oleochemicals has been actively pursued (1,2). Lipase-catalyzed reactions offer several benefits over chemically catalyzed reactions, such as milder operating conditions, cleaner products and reduced waste production (1,3). One attractive feature of lipases is the specificity of the enzyme with respect to glyceride positions and fatty acid types (4), which could seldom be achieved with chemical catalysts. 1,3-Positional specificity has been exploited in a number of applications to obtain high-valued speciality fats, such as cocoa butter substitutes (1,5,6) or hardened vegetable oils with butter-fat properties (1). A major problem of lipase-catalyzed reactions is the high cost of enzymes, but the reusability of immobilized lipases will make the enzymatic process more economical.

Hydrolysis of triglycerides into partial glycerides with 1,3-specific lipases is useful for ascertaining the nature and physical characteristics of fats and oils. Furthermore, analysis of the types of fatty acids at the 1,3-position is important because it is now known that fats modified chemically or enzymatically can have nutritional consequences, implying that the saturated/polyunsaturated nature of the 2-positional fatty acid may be crucial (7). Examples where nutritional properties have been altered or enhanced are the chemical interesterification of lard (8) and the enzymatic incorporation of eicosapentaenoic and docosahexaenoic acids into sardine oils (9). The present standard method for the analysis of fatty acids at the 1,3-position of triacylglycerols is by pancreatic lipase (10). As many 1,3-specific lipases are now well documented, it is useful to study the parameters that would affect their 1,3-specificity so that more applications may be made. This paper examines two fungal lipases from *Mucor miehei* and *Humicola lanuginosa* and the effect of hexane and diethyl ether on the 1,3-specificity.

EXPERIMENTAL PROCEDURES

Materials. Cocoa butter was a sample from the Palm Oil Research Institute of Malaysia (Bangi, Malaysia), soybean oil was from Econfood Manufacturing (M) Sdn. Bhd. (Pasir Gudang, Malaysia) and olive oil was from Sigma (St. Louis, MO). Three types of lipases have been used. *Mucor miehei* lipase, immobilized on a macroporous anion exchange resin (Lipozyme™ IM 20), was a gift from Novo Industri (Copenhagen, Denmark), and pancreatic lipase was purchased from Sigma. *Humicola lanuginosa* was cultured in a stirred-tank fermenter and the lipase was immobilized on Silica gel 60 (230–400 mesh, Merck, Darmstadt, Germany).

All solvents were of analytical grade and were used without further purification, except for diethyl ether, which was filtered through a short column of neutral alumina (activity I, Merck) prior to use. Silica gel 60 (230–400 mesh) for column chromatography and analytical thin-layer chromatography (TLC) plates precoated with Silica gel 60 F_{254} were purchased from Merck.

Immobilization of lipase. Silica gel 60 (2 g) was suspended in the crude culture filtrate of *Humicola lanuginosa* (200 mL, about 15°C). The mixture was stirred for 30 min after which it was filtered through a sintered-glass filter funnel (porosity 3). The immobilized lipase was washed with cold distilled water (3 × 100 mL), air-dried and kept in the freezer for future use.

Enzyme assays. The "transesterification activity" of each lipase was assayed by determining the amount of decyl palmitate produced from the reaction of tripalmitin and decanol at 55°C for 30 min. One unit (U) of "transesterification activity" is the amount of lipase required to produce 1 μmole decyl palmitate/min. Lipozyme has an activity of 172 U/g with 7% (w/w) moisture content. Immobilized lipase from *Humicola lanuginosa* has an activity of 3.3 U/g at 80% (w/w) moisture content.

Transesterification/alcoholysis. A sample of triglycerides (0.2 g) was dissolved in a solvent system of hexane/alkyl acetate or hexane/alcohol at a ratio of 9:1 (vol/vol). Lipozyme (0.4 g) was quickly added to the reaction mixture in a stirred bioreactor at 50°C for 3 h or until the degree of transesterification or alcoholysis required was attained. The reaction mixture was then filtered to remove the immobilized lipase. Esters were isolated by silica gel column chromatography with hexane/chloroform (1:1, vol/vol) as eluent. For alkyl acetates or alcohols with four carbons or more, an amount of 0.69 mmol alkyl acetates or alcohols and triglycerides (0.2 g) were dissolved in hexane (10 mL). Lipozyme (0.4 g) was premixed with water (10%, w/w) prior to use. For the immobilized *Humicola* lipase, the incubation time was extended to 24 h. For reactions with diethyl ether/alkyl acetate or diethyl ether/alcohol solvent system (9:1, vol/vol), the incubation temperature was 36°C and incubation time was 5 h for Lipozyme and 24 h for immobilized *Humicola* lipase, respectively. Reactions catalyzed by *Humicola* lipase in the diethyl ether solvent gave low yields of esters. In this case, the fatty acids were isolated by TLC in a solvent system of diethyl ether/petroleum ether/acetic acid (5:5:0.2, vol/vol/vol) and further esterified to methyl esters prior to gas chromatographic (GC) analysis. The products of

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transesterification/alcoholysis by Lipozyme were alkyl esters, whereas those from immobilized lipase of *Humicola lanuginosa* were fatty acids (Tables 1 and 2).

Chemical methanolysis of triglycerides was carried out with 1 M sodium methoxide in methanol. Hydrolysis catalyzed by pancreatic lipase was done according to the method described by Christie (10).

Product analysis. GC analyses of alkyl esters were performed on a Shimadzu GC 14A Gas Chromatograph (Kyoto, Japan) equipped with a glass column (2.1 m × 3 mm i.d.) packed with 10% SP-2330 on 100/120 Chromosorb R (Supelco, Bellefonte, PA) and fitted with a flame-ionization detector. The operating temperature was from 190 to 240°C, programmed at the rate of 2°C/min, with an initial 2-min hold and a final hold of 15 min. Nitrogen was used as the carrier gas. Reaction mixtures were also analyzed by the cool-on-column injection mode in an HP 5890 series II Gas Chromatograph (Hewlett-Packard, Avondale, PA) equipped with a capillary column of 25 m × 0.25 mm i.d. (Quadrex 007-65HT, New Haven, CT) with the film thickness of 0.1 μm. Nitrogen was used as the carrier gas at flow rate of 2 mL/min and made-up to 17 mL/min. The operating temperature was from 60 to 365°C, programmed at the rate of 10°C/min, with an initial hold of 1 min and a final hold of 10 min. All the chromatograms were recorded with an HP 3396A integrator.

^{13}C Nuclear magnetic resonance (NMR) were recorded on JEOL spectrometers, FX-100 (Japan) and GSX 270 FT (Japan), with deuterated benzene (Sigma) as external reference. Authentic standards (1,2-dipalmitin, 1,3-di-

TABLE 1

Transesterifications/Alcoholyses of Triglycerides with Hexane as Solvent

Substrate	Alkoxy donor	Catalyst	Time (h)	% Yield[a]	% Composition[b]					
					16:0	16:1	18:0	18:1	18:2	20:0
Cocoa butter	MeOAc	MM[c]	3	68	30.5	—	40.2	26.2	2.3	0.8
Cocoa butter	MeOAc	HL[c]	24	5	34.6	—	40.3	22.0	2.1	1.0
Cocoa butter	EtOAc	MM	3	74	30.5	—	38.8	27.0	2.7	1.1
Cocoa butter	EtOH	MM	24	86	26.6	—	35.2	34.2	3.1	0.9
Cocoa butter	BuOH	MM	5	86	27.2	—	34.5	34.2	3.2	0.9
Cocoa butter	Decanol	MM	5	82	28.7	—	34.4	32.7	3.0	1.1
Cocoa butter	Decyl acetate	MM	5	85	28.5	—	34.5	32.7	3.0	1.2
Cocoa butter	MeOH	NaOMe	—	—	26.5	—	34.6	34.8	3.1	0.9
Lit.[d]	—	—	—	—	24.0	0.4	35.0	36.0	3.4	1.1

[a] Yields are based on 1 mole of triglyceride giving 3 moles of fatty esters; hexane, 50°C.

[b] % Area is based on gas chromatographic flame-ionization detector response.

[c] MM, *Mucor miehei*; HL, *Humicola lanuginosa.*

[d] Literature data of total fatty acid composition (Ref. 11).

TABLE 2

Transesterifications/Alcoholyses of Triglycerides with Diethyl Ether as Solvent

Substrate	Alkoxy donor	Lipase	Time (h)	% Yield[a]	% Composition[b]						
					16:0	16:1	18:0	18:1	18:2	18:3	20:0
Cocoa butter	MeOAc	MM[c]	5	39	36.9	—	50.2	10.7	0.8	—	1.3
Cocoa butter	EtOAc	MM	5	49	37.2	—	50.7	9.9	0.9	—	1.3
Cocoa butter	BuOAc	MM	5	49	38.1	—	48.5	11.0	1.1	—	1.3
Cocoa butter	BuOH	MM	2	70	38.7	—	50.0	9.1	1.0	—	1.3
Cocoa butter	MeOAc	HL[c]	24	53[d]	41.9	—	52.5	4.0	0.3	—	1.3
Cocoa butter	EtOAc	HL	24	48[d]	43.3	—	52.0	3.6	—	—	1.1
Cocoa butter	BuOAc	HL	24	81[d]	41.4	—	54.9	2.3	0.1	—	1.2
Cocoa butter	H_2O	PL[c]	0.25	—	36.8	—	50.9	10.4	0.8	—	1.1
Cocoa butter	—	—	—	—	[e]35.2	0.4	51.5	10.4	0.8	—	1.6
FAME composition of 2-MG[f]					4.0	—	3.8	85.6	6.6	—	—
2-MG of cocoa butter					[e]1.7	0.2	2.1	87.4	8.6	—	—
Soybean	MeOAc	MM	5	33	17.5	—	6.0	23.5	46.8	6.2	—
Soybean	—	—	—	—	[e]13.4	—	5.7	25.4	46.7	8.7	—
Olive oil	MeOAc	MM	5	33	14.6	0.5	4.4	76.1	3.9	0.4	—
Olive oil	—	—	—	—	[e]14.9	0.8	3.4	72.5	7.4	0.9	—

[a] Yields are based on 1 mole of triglyceride giving 2 moles of fatty esters/acids; diethyl ether, 36°C.

[b] % Area is based on gas chromatographic flame-ionization detector response.

[c] MM, *Mucor miehei;* HL, *Humicola lanuginosa;* PL, pancreatic lipase.

[d] Yield of fatty acids.

[e] Literature data of fatty acid composition of the 1,3- and 2-positions of the triglycerides (Ref. 11).

[f] 2-Monoglyceride (MG) isolated from the reaction of cocoa butter with MeOAc catalyzed by *Humicola* lipase. FAME, fatty acid methyl ester.

palmitin, 1-monopalmitin and 2-monopalmitin) were purchased from Sigma. Quantitation of ^{13}C NMR samples was carried out by gated decoupling and 10-s delay time because of long and unequal relaxation times, but calibrations were still necessary. Products were determined from the following chemical shifts in C_6D_6: Triglycerides (TG) at δ 173.51 (OC=O), 173.20 (OC=O), 69.71 (CHO) and 62.89 ppm (CH_2O); 1,2-diglycerides (DG) at δ 173.99 (OC=O), 173.53 (OC=O), 72.86 (CHO), 63.38 (CH_2OH) and 61.67 ppm (CH_2O); 2-monoglycerides (MG) at δ 174.12 (OC=O), 75.67 (CHO) and 62.22 ppm (CH_2OH); fatty acids at δ 178.90 ppm (OC=O) and methyl esters at δ 173.64 (OC=O) and 51.43 ppm (CH_3O).

RESULTS AND DISCUSSION

Cocoa butter contains a large percentage of dipalmitoyl-oleoyl-glycerol (POP), palmitoyl-oleoyl-stearoyl-glycerol (POS) and distearoyl-oleoyl-glycerol (SOS) (glycerides include: 1,2-dioleoyl-3-linoleoyl-glycerol, 0.2%; palmitoyl-oleoyl-linoleoyl-glycerol, 2.4%; 1,2-dioleoyl-3-palmitoyl-glycerol, 2.2%; POP, 18.9%; 1,2-dioleoyl-3-stearoyl-glycerol, 2.4%; POS, 41.3% SOS, 29.7% and tristearin, 1.9%) (6). It is a convenient substrate for the determination of 1,3-specificity in lipases. A 1,3-specific lipase will favorably hydrolyze the fatty acids (predominantly palmitic and stearic) from positions 1 and 3 in cocoa butter, leaving the predominant oleate unaffected at position 2. Results of regiospecificity studies with *Mucor miehei* and *Humicola lanuginosa* lipases in two organic solvent systems, hexane and diethyl ether, are given in Tables 1 and 2, respectively.

The observed product distributions of the fatty acids (after conversion to fatty acid methyl esters, FAME) or fatty alkyl esters, as analyzed by gas chromatography, showed that the reactions could be solvent-dependant. When hexane was used as a solvent, the data obtained indicate that lipases of *Mucor miehei* and *Humicola lanuginosa* were apparently not 1,3-specific (Table 1). Large amounts of oleic acid (18:1) were liberated and exceeded those reported in the literature (11), indicating that some of the fatty acids from position 2 have also reacted. If the reaction time of transesterification was prolonged, *Mucor miehei* lipase will eventually appear to be non-specific (3). Berger and Schneider (12) commented that the lipase of *Mucor miehei* is not strictly 1,3-specific, but did show a distinct 1,3-selectivity toward glycerides because the incubation of 1,3-dilaurin in butyl methyl ether gave 16.3% 1,2-dilaurin after 72 h.

Different results were obtained when the lipase-catalyzed reactions were carried out with diethyl ether as solvent. In this system, *Mucor miehei* lipase clearly demonstrates 1,3-specificity (Table 2). 2-Monoglycerides, shown in the GC chromatogram along with the fatty acids hydrolyzed from positions 1 and 3, were indicative of 1,3-specific products. The regiospecificity in the products was verified by ^{13}C NMR spectroscopy, partial glycerides formed being only 2-monoglycerides (MG), 1,2-DG and 2,3-DG. Comparable results were also obtained from the pancreatic lipase-catalyzed hydrolysis.

The *Humicola* lipase-catalyzed reactions gave results that are also indicative of 1,3-specificity, but the data were slightly different from those obtained with pancreatic lipase. This was later determined to be due to the effect of chain length specificity, as *Humicola* lipase has a greater affinity for C_{16}, as compared to C_{18}, fatty acids, resulting in the more rapid formation of the palmitic acid, which is evident in the early stages of the reaction. However, 1,3-specificity of this lipase is strongly supported by the detection and isolation of 2-MG but not 1-MG (Table 2).

^{13}C NMR spectroscopy could be readily used to confirm the presence of partial glycerides in the reaction mixture. The characteristic chemical shifts of the glycerol carbons are: (C_6D_6) 2-MG at δ 75.67 (CHO) and 62.22 ppm (CH_2OH) and 1,2-DG or 2,3-DG at δ 72.86 (CHO), 63.38 (CH_2OH) and 61.67 ppm (CH_2O) under conditions for quantitation. The assignments of resonances were made by comparison with authentic standards.

Most of the data presented for transesterification or alcoholysis reactions in hexane (Table 1) showed that considerable amounts of the triglycerides had reacted. The ester yield was more than what could be accounted for if these lipases are 1,3-specific, indicating that some of the fatty acids at position 2 have also hydrolyzed subsequent to acyl migration. The appearance of apparent non-1,3-specific products means that significant acyl migration has occurred in the hexane medium. In the earlier stages of reaction, for example in the first 5 min, a small amount of 1,3-specific products was detectable (Table 3). As the reaction time was extended to 3 h, the relative percentage of oleic (18:1) and linoleic (18:2) acids increased significantly, resulting from hydrolysis subsequent to a 2- to 1(3)-acyl shift. However, reactions carried out in diethyl ether under similar conditions provided percentages of fatty acid products that remained fairly constant throughout the course of reaction. The FAME composition obtained was similar to that of pancreatic lipase-catalyzed hydrolysis, which indicates that 1,3-specific analysis of fatty acids in TG could be conveniently carried out with the readily available immobilized lipase Lipozyme.

Previous reports have shown that the presence of moisture (13), acid (14) and silica gel (15) may induce high amounts of acyl migration. Spontaneous acyl migration will invariably occur in the transesterification system because the immobilization matrix of *Mucor miehei* and *Humicola lanuginosa* lipases contain 7 and 80% moisture, respectively. Free fatty acids, acetic acid (from ROAc substrates) and silica gel (particulate matrix of *Humicola* lipase) could be other contributing factors to catalytic acyl migration. One possible reason for the higher yields of acyl migration observed in hexane is probably due to the partitioning of free fatty acids present together with the relatively polar 2-MG (and 1,2-DG) into the microaqueous phase of the immobilization matrix, thus providing favorable conditions for acid-catalyzed acyl migration. Studies by Heisler *et al.* (13) indicate that although acyl migration is spontaneous, it may also be enzymatic. When the solvent system was changed to diethyl ether, less of the products from competitive acyl migration were observed. The free fatty acids and partial glycerides are more soluble in diethyl ether than in hexane, and thus the possibility of 2- to 1(3)-acyl shifts in the immobilization (aqueous) matrix is reduced. It is probably due to the same reason that lipase of *Candida cylindracea*, a well-known non-specific lipase, has been reported to be 1,3-specific in the presence of 20% ethanol (16). The moisture content of the immobilization matrix of *H. lanuginosa* was 80% and was

TABLE 3

A Time Course Reaction of Transesterifications in Hexane and Diethyl Ether

Lipase	Reaction medium	Time	Reaction mixture composition (%)[a]					FAME composition (%)[a]					
			ME	FA	MG	DG	TG	16:0	16:1	18:0	18:1	18:2	20:0
MM[b]	Hexane/MeOAc	5 min	15.0	16.2	4.7	3.7	60.3	36.7	—	49.3	11.9	1.0	1.0
		15 min	48.9	7.1	9.7	9.2	25.0	36.5	—	47.2	12.8	1.2	2.3
		1 h	77.0	—	14.8	6.2	2.0	33.4	—	44.6	18.3	2.2	1.4
		3 h	87.5	—	11.3	1.2	—	30.5	—	40.2	26.2	2.3	0.8
	MeOH/NaOMe							26.5	—	34.6	34.8	3.1	0.9
MM	Ether/MeOAc	30 min	5.3	17.2	2.5	2.8	72.1	38.1	—	50.9	9.3	1.0	0.7
		2 h	47.1	15.8	13.7	4.9	18.4	37.9	—	50.7	8.8	1.6	1.0
		5 h	78.0	—	17.4	2.9	1.7	36.9	—	50.2	10.7	0.8	1.3
PL[b]	H_2O							36.8	—	50.9	10.4	0.8	1.1

[a] % Area from gas chromatographic flame-ionization detector response; ME, methyl esters; FA, fatty acids; MG, monoglycerides (1- and 2-MG inclusive); DG, diglycerides (1,2- and 1.3-DG inclusive); TG, triglycerides; cocoa butter was used as substrate. FAME, fatty acid methyl ester.

[b] MM, *Mucor miehei;* PL, pancreatic lipase.

much higher compared to Lipozyme (7% moisture content). Lyophilization of the former biocatalyst to a lower moisture content has resulted in loss of enzyme activity. Although the moisture content of the biocatalyst is high, the available water molecules required for lipase transesterification activity may be low, due to high water adsorption of the silica gel matrix as compared to the support matrix of Lipozyme, which is a macroporous anion exchange resin. It is likely that specific requirements of water molecules by individual lipases are crucial in the regiospecificity or otherwise of their reactions, and the water content within the lipases can be influenced indirectly by the solvent.

In conclusion, the use of readily available immobilized 1,3-specific lipase for transesterification and alcoholysis reactions in diethyl ether provides a convenient analytical methodology in combination with GC and NMR spectroscopy for the determination of fatty acid distribution with triglycerides.

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