Use of *Rhizopus delemar* Lipase as Compared with Other Lipases for Determination of *sn*-2 Fatty Acids in Triacylglycerol

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ABSTRACT: The FA composition in the *sn*-2 position of TAG is routinely determined after porcine pancreatic lipase hydrolysis. However, the content of saturated FA increased when a pancreatic lipase preparation with higher specific activity was used. Lipase from *Rhizopus delemar* was selected as a potential replacement lipase for the following reasons: (i) The FA specificity is nearly equivalent in hydrolysis activity toward FA such as lauric, myristic, palmitic, palmitoleic, stearic, oleic, linoleic, and α-linolenic acids; and (ii) lipase from *R. delemar* hydrolyzes fatty acyl residues at the *sn*-1,3 positions of TAG. Acyl migration products were present at less than 0.8% in lipase hydrolysates containing 6–14% of *sn*-2 MAG. A reproducibility CV of less than 5% was obtained in a collaborative study in which the compositions of the main FA at the *sn*-2 position in olive oil were determined using lipase from *R. delemar*.

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KEY WORDS: Acyl migration, collaborative study, fatty acid composition, lipase, monoacylglycerol, pancreatic lipase, *Rhizopus delemar, sn*-2 position, triacylglycerol.

The FA position in dietary TAG has an effect on lipid metabolism. Hydrolyses of *sn*-1- and *sn*-3-position FA in TAG by pancreatic lipase (1) produce *sn*-2 MAG. Many studies have now focused on the importance of the FA at the *sn*-2 position (2–6). Better absorption and effects on metabolism of palmitic acid (2–4) and linoleic acid (5) at the *sn*-2 position were reported. The FA composition at the *sn*-2 position should be considered in the design of structured TAG (6). The distribution of FA in *sn*-2 MAG has been routinely determined after porcine pancreatic lipase (PPL) hydrolysis (6–9). The *sn*-2 MAG formed by PPL hydrolysis was also used to determine FA at the *sn*-2 position in a previous collaborative study of the Japan Oil Chemists' Society (Nakasato, S., and H. Seino, unpublished results). This paper cited in part the previous result that saturated FA in a *sn*-2 position increased on using the PPL preparation with higher specific activity. Chemical degradation with a Grignard reagent (9–11) has been conducted to test TAG containing FA resistant to hydrolysis by lipase, such as long-chain PUFA. Still, a simple, safe, and environmentally friendly procedure using a lipase remains to be established. Nonenzymatic acyl migration occurs when a polar substance, such as water, is present in an assay system (12). The time required for the lipase reaction and preparation of the *sn*-2 MAG with TLC should thus be made as short as possible to minimize acyl migration. In a collaborative study by the Japan Oil Chemists' Society, the FA composition in the *sn*-2 position of beef tallow was determined by using lipase from *Rhizopus delemar* (12). The present paper describes comparisons of lipases and characteristic properties of lipase from *R. delemar* for FA determination at the *sn*-2 position in TAG.

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MATERIALS AND METHODS

Materials. Olive oil was obtained from Wako Pure Chemicals (Osaka, Japan). Lipase from *R. delemar* (Fine Grade) was from Seikagaku Corporation (Tokyo, Japan); the lipase preparation contained at least 600 units/mg protein, the level that is ordinarily used in *sn*-2 FA analysis. Lipase from *R. delemar* (Talipase, equivalent to T"Amano"), formerly from Tanabe Seiyaku Co., Ltd. (Osaka, Japan), is now produced by Amano Enzyme (Nagoya, Japan) and has a specific activity of approximately 10 units/mg protein. Lipase from *Alcaligenes* sp. (PL) and cocoa butter were from Meito Sangyo Co., Ltd. (Tokyo, Japan). PPL (Type II) was from Sigma (St. Louis, MO), with a specific activity of 100–400 units/mg protein. Pancreatin was from Wako Pure Chemicals, with a specific activity of 10–40 units/mg protein. 1,3-Distearoyl-2-oleoylglycerol (SOS, 99+%) was purchased from Sigma. The Chromarod S-III was from Iatron Lab. Inc. (Tokyo, Japan).

Analysis of sn*-2 FA in cocoa butter with PPL.* The reaction mixture, which contained 0.3 g cocoa butter, 1.5 mL 0.1% sodium cholate solution, 0.6 mL 22% calcium chloride solution, and 6 mL pancreatic lipase in Tris buffer at pH 8, was shaken at 40°C for 2 min to produce *sn*-2 MAG. The hydrolysate was separated by TLC on a silica gel plate impreg-

nated with 3% boric acid. The developing solvent for the TLC was chloroform/acetone (96:4). The *sn*-2 MAG fraction was converted to methyl esters with boron trifluoride/methanol (13) and analyzed by GC equipped with a packed column and FID (14). The concentration of lipase was adjusted to obtain an acid value of 50–70 with the reaction product just described.

FA specificity of lipase determined with randomized ester. Quantities of trilaurin, trimyristin, tripalmitin, tristearin, olive oil, soybean oil, and linseed oil were mixed so that FA containing 12–18 carbon atoms would be present in fairly significant amounts. The mixture was dried over 3 Å molecular sieves *in vacuo* overnight. To 10 g of the mixed oil, 0.2% sodium methoxide was added, and the system was stirred at 70°C for 8 h. Randomized esterification was terminated by addition of diluted citric acid. FFA were eliminated by three washes with dilute ammonia water. The system was washed with water and dried over 3 Å molecular sieves under vacuum overnight. The ester thus obtained was used as substrate for lipases. The randomized ester was hydrolyzed for 1 min with several different lipases. The FA compositions in FA fractions of hydrolysate formed by lipase were compared to the FA composition of randomized ester.

TLC–FID analysis of hydrolysate formed by lipase. This analysis was conducted using a thin-layer chromatograph-hydrogen FID (TLC–FID) with an Iatroscan TH 10 (Iatron Lab). The sample was applied on chromarod S-III and developed in benzene/chloroform/acetic acid (50:20:0.7). During sample analysis 1-MAG and 2-MAG, or 1,2-DAG and 1,3-DAG were separated following application on the chromarod S-III treated with 3% boric acid and development with two different solvents. The first development was carried out to 8 cm in pure chloroform and the second to 10 cm with chloroform/methanol/ammonia (70:0.04:0.01).

RESULTS AND DISCUSSION

Analysis of sn*-2 FA with pancreatic lipase.* In the previous Japan Oil Chemists' Society committee held on November 25, 1986, pancreatic lipase was used to determine FA at the *sn*-2 position (Nakasato, S., and H. Seino, unpublished results). The results for levels of saturated FA such as palmitic acid (C16:0) and stearic acid (C18:0) in the *sn*-2 FA of cocoa butter were different when pancreatic lipases from different makers were used (Table 1). The specific activity of lipase from Sigma is 100 times that of pancreatin from Wako Pure Chemicals. The saturated FA content increased when pancreatic lipase with a higher specific activity was used. On activation of pancreatic lipase by bile salt micelles and colipase or by a lipid–water interface, the lid that covers the active site of the lipase moves to open (15). Then pancreatic lipase and colipase create a large hydrophobic plateau that can interact with lipid–water interfaces (15). The cofactor content may have depended on the purity of the pancreatic lipases. Pancreatic lipase is ideal for the study of positional FA function because lipolysis in the intestine is the first step in lipid metabolism. Further studies are necessary to determine whether lipase preparation from the intestine should be used to investigate the physiological roles of

TABLE 1
Collaborative Analysis for *sn*-2 FA in Cocoa Butter with Pancreatic Lipase

Laboratory	Entry	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Results (%) with lipase from Sigma (St. Louis, MO)							
	a	2.3	0.3	3.7	84.7	8.6	0.4
1	b	3.7	0.3	3.9	82.4	9.3	0.4
	a	1.65		1.84	86.38	9.26	
2	b	1.63		1.89	86.21	9.37	
	a	4.1	0.6	3.1	81.6	10	0.6
3	b	2.5	0.5	3.3	82.1	10	0.6
	a	2.7	0.4	2.9	85.3	8.2	0.5
4	b	3.1	0.4	2.9	84.6	8.8	0.6
	a	2.9	0	4.6	82.9	8.9	0.2
5	b	3.2	0	4.6	81.9	9.7	0
Mean values		2.8	0.4	3.3	83.8	9.2	0.4
Reproducibility (CV)		29	83	31	2.29	6.8	56
Results (%) with pancreatin from Wako (Osako, Japan)							
	a	2.7	0.3	3.1	84.2	9.3	0.4
1	b	1.8	0.3	3.3	85.2	9	0.4
	a	1.94		1.87	85.56	9.51	
2	b	2.27		2.38	84.54	9.33	
	a	1.9	0.4	2	85.7	9.7	0.3
3	b	1.8	0.4	1.9	85.6	9.9	0.4
	a	1.9	0.5	2.6	86.7	7.9	0.4
4	b	1.6	0.3	2.3	86.9	8.2	0.7
	a	1.4	0.3	1.8	85.9	9.6	0
5	b	1.4	0.2	1.9	86.5	9.4	0
Mean values		1.9	0.3	2.3	85.5	9.2	0.3
Reproducibility (CV)		22	28	24	0.884	7.4	72

TABLE 2
Comparison of FA Specificity Using Randomized Ester

		Hydrolysate			
Lipase origin		*R. delemar*	*Achromobacter*	*Alcaligenes*	*Candida*
FFA		32.2%	21.8%	36.4%	32.5%
Correlation coefficients[a] between FA composition of randomized ester and FA composition of hydrolysate					
	1.00	0.953	0.940	0.781	0.593
	Randomized ester				
C12:0	7.26%	9.34%	5.62%	5.11%	7.34%
C14:0	10.69	12.53	8.84	7.97	9.43
C16:0	18.84	18.35	24.26	27.4	16.26
C18:0	17.57	16.59	17.75	12.15	11.27
C18:1	15.84	15.37	13.38	11.73	15.76
C18:2	15.64	14.51	16.37	18.43	18.82
C18:3	14.17	12.72	13.28	17.21	21.12

[a]Correlation coefficient = $[\Sigma(X - M_X)\ (Y - M_Y)]/[\Sigma(X - M_X)^2]^{1/2}\ [\Sigma(Y - M_Y)^2]^{1/2}$, where M_X is the mean value of FA composition (X) of randomized ester, M_Y is the mean value of FA composition (Y) of hydrolysate. *R. delemar* = *Rhizopus delemar*.

FA. Recently, it was reported that human dietary fat is sequentially hydrolyzed by two main enzymes, human gastric lipase and human pancreatic lipase (16).

Lipase for lipolysis product of FA with 12–18 carbon atoms. The fatty acyl residue specificity of lipases was compared with the hydrolysate following hydrolysis of randomized esters for 1 min with the lipases listed in Table 2. The amount of FFA hydrolyzed from random esters by the lipase from *R. delemar* was 32.2%. The FA composition of the hydrolysate obtained with lipase from *R. delemar* resembled the FA composition of the randomized ester, with the highest correlation coefficient (0.953) between FA compositions of hydrolysate and FA compositions of randomized ester obtained in this study. The compositions of hydrolysates obtained with *Achromobacter* sp. lipase were also very similar to that of the randomized ester, although the hydrolysis ratio did not increase. A lipase preparation with high activity is necessary to decrease reaction time. When moisure is present with acylglycerol, acyl migration readily occurs, as reported previously (12). The relative FA activity of lipase from *R. delemar* toward the esters of oleic acid (18:1), palmitoleic acid (16:1), γ-linolenic acid (18:3n-6), arachidonic acid (20:4), EPA (20:5), and DHA (20:6) was previously reported to be 100, 105, 19, 34, 35, and 10, respectively (17).

Selection of lipase for the hydrolysis of sn-*1,3 fatty acyl residues*. The substrate SOS was hydrolyzed by lipases from *Alcaligenes* sp. and *R. delemar*. No 1,3-DAG were detected in the reaction products obtained with *R. delemar* lipase (Table 3). The stearic acid content in the FA fraction obtained with *R. delemar* lipase was 99.55%, and the oleic acid content in the MAG fraction obtained with *R. delemar* lipase was 98.31%. *Rhizopus delemar* lipase was thus shown to hydrolyze fatty acyl residues at *sn*-1,3 of TAG. Results using 1,3-dipalmitoyl-2-oleoylglycerol, 1,2-dioleoyl-3-palmitoylglycerol, and 1-palmitoyl-2-oleoyl-3-stearoylglycerol as substrates indicated the same positional specificity.

Effect of acyl migration. Acyl migration products from TAG hydrolyzed by *sn*-1,3-specific lipases are 1(3)-MAG and 1,3-DAG. Fewer of these acyl migration products were obtained with *R. delemar* lipase than with *Alcaligenes* lipase. The acyl migration products were less than 0.8% in the *R. delemar* lipase hydrolysate at levels of 6–14% of *sn*-2 MAG (Table 4, part A).

TABLE 3
Selection of Lipase to Hydrolyze *sn*-1,3 Fatty Acyl Residues

		Product from 1,3-distearoyl-2-oleoylglycerol				
Lipases used		TAG	FA	1,3-DAG	1,2-DAG	MAG
Alcaligenes sp.		35.8%	24.2%	0.2%	28.5%	11.3%
R. delemar[a]		43.2	19.9	0	27.1	9.7
FA compositions in above fractions						
Fraction	Lipase used		C16:0	C18:0	C18:1	
MAG	*Alcaligenes* sp.		0.63%	3.23%	96.14%	
	R. delemar		0.7	0.98	98.31	
FA	*Alcaligenes* sp.		0.14	97.89	1.97	
	R. delemar		0.13	99.55	0.32	

[a]*Rhizopus delemar*.

TABLE 4
Analysis of *sn*-2 FA in TAG with Lipase from *Rhizopus delemar*

A. Acyl migration products in cocoa butter hydrolysate						
Lipases used	TAG	FA	1,3-DAG	1,2-DAG	2-MAG	1-MAG
5% Talipase from *R. delemar*	42.4%	24.8%	0.8%	25.5%	6.3%	0.1%
10% Talipase from *R. delemar*	33.2	34.1	0.5	18.6	13.5	0.1
B. FA in *sn*-2 position of cocoa butter with different purity lipases						
Lipases used	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
2% Fine Grade	3.9 ± 0.3%	0.00%	4.3 ± 0.3%	84.8 ± 0.3%	6.8 ± 0.1%	0.08 ± 0.07%
20% Talipase	4.4 ± 0.5	0.00	4.1 ± 0.5	84.8 ± 0.9	6.7 ± 0.1	0.00

TABLE 5
Collaborative Analysis for *sn*-2 Positional FA in Olive Oil

Laboratory	Entry	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
	a	0.7%	0.6%	0.2%	86.2%	10.7%	0.8%
1	b	0.9	0.6	0.5	85.1	10.6	0.7
	a	0.7	0.6	0.2	86.5	11.2	0.8
2	b	0.7	0.6	0.1	86.5	11.1	0.9
	a	0.8	0.7	0.2	86.2	11.3	0.9
3	b	0.8	0.7	0.2	86.4	11.2	0.8
	a	1.0	0.8	0.2	85.1	11.9	1.1
4	b	1.3	1.4	0.3	83.5	12.2	1.1
	a	0.9	0.7	0.0	84.5	11.5	0.9
5	b	0.9	0.7	0.2	84.5	11.4	1.0
	a	0.6	0.8	0.2	86.2	11.2	0.8
	b	0.9	0.7	0.2	86.0	11.2	0.7
6	a	1.2	0.7	0.3	88.5	7.5	0.4
	b	1.1	0.7	0.3	88.2	7.6	0.5
Number (n) of laboratories subsequent to elimination of outliers							
		7	6	7	7	6	7
Mean values (M) of *sn*-2 positional FA composition							
$M = \Sigma(a + b)/2n$		0.9%	0.7%	0.2%	86.0%	11.3%	0.8%
Repeatability SD							
$Sr = (\Sigma(a - b)^2/2n)^{1/2}$		0.1	0.03	0.1	0.53	0.1	0.07
Repeatability coefficients of variation (CVr)							
$CVr = 100(Sr/M)$		14	4	47	17	0.921	8
Repeatability							
$r = 2.83Sr$		0.4	0.08	0.3	1.51	0.294	0.2
Reproducibility SD[a]							
$S_R = (S_L{}^2 + Sr^2)^{1/2}$		0.2	0.07	0.1	1.41	0.461	0.2
Reproducibility coefficients of variation CV_R							
$CV_R = 100(S_R/M)$		23	10	51	1.65	4.08	23
Reproducibility							
$R = 2.83\ S_R$		0.6	0.2	0.3	4	1.3	0.5

[a]Where $SL^2 = [\{n\Sigma[(a + b)^2/4] - [\Sigma(a + b)/2]^2\}/[n(n - 1)]] - \{[\Sigma(a - b)^2]/[4n]\}$.

Analysis of the sn-*2 FA composition of cocoa butter with* R. delemar *lipases*. For this analysis, lipases from two different manufacturers were used: Fine Grade lipase and Talipase. The former is 60 times higher in specific activity than the latter. The FA compositions found in the *sn*-2 position of cocoa butter were the same within experimental error (Table 4, part B). The levels of C16:0, C18:0, and C18:2 FA in the *sn*-2 position of cocoa butter obtained with pancreatic lipase from Sigma (Table 1, upper mean values) and the same levels obtained with Fine Grade lipase from *R. delemar* (Table 4, part B), were 2.8, 3.3, and 9.2% and 3.9, 4.3, and 6.8%, respectively. Thus, the lipase from *R. delemar* released more saturated FA and less linoleic acid than the PPL. Although the specificity of lipase from *R. delemar* was not the same as PPL, the lipase can be used to determine *sn*-2 FA in TAG. The lipase from *R. delemar* displayed high activity in the hydrolysis of lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1) stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α-linolenic acid (18:3n-3) in the 1- and 3-positions of TAG.

Collaborative study of the sn-*2 FA of olive oil.* Conversion of the *sn*-2 FA in MAG to methyl esters was carried out with sodium methoxide, not boron trifluoride/methanol. A one-step conversion to methyl esters was possible by transesterification with sodium methoxide, whereas saponification to decompose the MAG and then methyl ester synthesis with boron trifluoride/methanol requires two steps (13). Boron trifluoride is toxic to the environment; sodium methoxide easily decomposes on contact with moisture; conversion to methyl esters was thus conducted with sodium methoxide from an ampoule (12). A collaborative study to determine *sn*-2 FA in olive oil was conducted using lipase from *R. delemar* with the procedure previously reported (12). As shown in Table 5, the reproducibility CV_R of the major FA, C18:1 and C18:2, were 1.65 and 4.08%, respectively.

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