Positional Specificities of Four Kinds of Microbial Lipases†

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Lipases from *Aspergillus niger* and *Rhizopus delemar* hydrolyzed triolein and produced 1,2(2,3)-diolein and 2-monoolein. These two lipases appear to have strong specificity towards the outer chains of the triglyceride. Comparing the proportions of fatty acids in position 1 (3) of cocoa butter with proportions of fatty acids liberated after limited hydrolysis of cocoa butter, it becomes clear that these two lipases do not hydrolyze the ester bond in position 2 of the triglyceride.

On the other hand, lipases from *Geotrichum candidum* Link and *Penicillium cyclopium* Westring attacked the fatty acid chains regardless of their positions. *Geotrichum candidum* lipase liberated oleic acid and palmitic acid in preference to stearic acid from cocoa butter.

A number of experiments have demonstrated that pancreatic lipase (EC 3.1.1.3) displays a strong specificity towards the outer chains of the triglyceride.^{1~5)} In studies on lipase from *Rhizopus* (*Rh.*) *arrhizus*, the lipase was shown to exhibit the same mode of action on triglyceride as that of pancreatic lipase.^{4,6~8)} Alford *et al.*⁹⁾ demonstrated that the lipase from *Geotrichum* (*Geo.*) *candidum* had strong specificity for unsaturated fatty acids regardless of their positions in the triglyceride. However, studies on the action patterns of purified microbial lipases have been insufficient.

In our laboratory, the lipases from *Aspergillus* (*Asp.*) *niger*,¹⁰⁾ *Rh. delemar*,^{11,12)} *Geo. candidum* Link¹³⁾ and *Penicillium* (*Pen.*) *cyclopium* Westring¹⁴⁾ were isolated as homogeneous states.

Previous studies have shown that the physicochemical properties and the substrate specificities of these lipases were distinct from each other.¹⁵⁾ Experimental data in our previous studies, however, seemed to suggest that the positional specificities of these lipases were different from each other. The present investigation was undertaken to clarify the positional specificities of four kinds of lipases using triolein, diolein and cocoa butter as

substrates.

MATERIALS AND METHODS

Lipase preparations. Homogeneously isolated lipases from *Asp. niger*,¹⁰⁾ *Rh. delemar* (C-lipase),¹²⁾ *Geo. candidum*¹³⁾ and *Pen. cyclopium* (A-lipase)¹⁴⁾ were used for the experiments.

Lipase activity. Lipase activity was assayed as described previously.¹⁰⁾ The activity which liberated a micro mole equivalent of fatty acid from olive oil in a minute was defined as 1 unit (U).

Substrates. Triolein and diolein were obtained from commercial triolein (Tokyo Kasei Kogyo Co.) by silica gel column chromatography.¹⁶⁾ Triolein was obtained as a homogeneous preparation, and diolein was a mixture of 78% of 1,3-diolein and 22% of 1,2(2,3)-diolein. Cocoa butter received from Okamura Seiyu Co. was purified according to the method of Quinlin and Weiser.¹⁶⁾ The purified preparation contained neither partial glycerides nor free fatty acids. The fatty acid distribution in the triglycerides of the cocoa butter employed was previously confirmed by use of pancreatic lipase (Difco Laboratories Co.). The result (Table I) was almost the same as that reported by Brockerhoff and Yukowski.¹⁷⁾

Reaction mixtures. The reaction mixture containing 0.5 g of glyceride, 4 ml of 0.05 M acetate buffer (pH 5.6), 1 ml of 0.1 M CaCl₂ solution and 0.1 ml of lipase solution (33U) was incubated at 30°C for 15~120 min with constant agitation (500 rpm) by a magnetic stirrer. After incubation, 25 ml of ethyl ether was added to the reaction mixture to stop the reaction and to extract the reaction products.

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Extraction of reaction products. From each reaction mixture, the products were extracted with ethyl ether and then separated from each other by thin-layer chromatography (TLC) as described below. The extraction was conducted rapidly, and the extracts were kept in a freezer until use in TLC to prevent spontaneous isomerization of the partial glycerides.

Thin-layer chromatography. Two kinds of TLC plates were selectively used in the experiment according to the purpose. For quantitative estimation by a densitometer, a precoated silica gel plate (type 60, Merck) was used. The developing solvent system was ligroin/ethyl ether/acetic acid (70:30:1). Boric acid impregnated silica gel plate was prepared to separate 1(3)-monoglyceride and 2-monoglyceride. The plate was developed in chloroform/acetone (96:4). The spots of hydrolysis products from triolein and diolein were visualized by spraying 50% sulfuric acid and heating at 150°C for 20 min.

Hydrolysis products of cocoa butter were separated by TLC using boric acid impregnated silica gel plates. They were visualized by spraying 2,7-dichlorofluorescein and UV light. The zone of each product was scraped off, and the product was extracted with ethyl ether.

Quantitative estimation of products. The charred spots were measured with a densitometer (Chromoscan, Joyce Loebl & Co. Ltd.) by reflectance scanning. This method revealed the relationship between the amount of spotted sample and light absorbancy, as shown in Fig. 1. The calibrated curve was also applicable for estimating the amounts of oleyl glyceride and oleic acid.

FIG. 1. Standard Calibration Curve of Oleyl Glyceride.

Experiment is described in detail in the text.

○, triolein; △, monoolein; ●, oleic acid.

FIG. 2. Hydrolysis Products of Triolein by Four Lipases.

TLC in chloroform/acetone (96:4) on boric acid impregnated silica gel plate. TO, triolein; 1,3-DO, 1,3-diolein; 1,2(2,3)-DO, 1,2(2,3)-diolein; OA, oleic acid; 2-MO, 2-monoolein; 1(3)-MO, 1(3)-monoolein.

FIG. 3. Hydrolysis Products of Diolein Mixture by Four Lipases.

TLC in ligroin/ethyl ether/acetic acid (70:30:1) on Silica Gel 60 plate (Merck). TO, triolin; OA, oleic acid; 1,3-DO, 1,3-diolein; 1,2(2,3)-DO, 1,2(2,3)-diolein; 1(3) and 2-MO, 1(3) and 2-monoolein.

TABLE I. FATTY ACID DISTRIBUTION IN TRIGLYCERIDES OF COCOA BUTTER

Cocoa butter and its hydrolysis products by the action of pancreatic lipase were assayed by use of TLC and GLC, and the fatty acid compositions were determined.

Fatty acid	Triglyceride	Mole % of fatty acids	
		Position in triglyceride	
		1(3)	2
Palmitic	28.0	36.8	3.8
Stearic	32.9	53.5	1.7
Oleic	36.1	9.1	85.8
Linoleic	2.7	0.1	8.2

Gas liquid chromatography. The fatty acid distribution of the cocoa butter and its enzymatic hydrolyzates were assayed by gas liquid chromatography (GLC). Liberated fatty acids and fatty acids formed by saponification of the glycerides were esterified with diazometane and assayed by GLC with a column

packed with 15% ethylene glycol succinate on Chromosorb W at 170°C.

Percentage of hydrolysis. To follow the percentage of hydrolysis of the substrate, the liberated fatty acids were titrated with potassium hydroxide. The percentage of hydrolysis was calculated on the basis of the saponification value of the substrate.

RESULTS

Hydrolysis of triolein

TLC of hydrolysis products of triolein by four lipases are shown in Fig. 2. Neither 1(3)-monoolein nor 1,3-diolein was accumulated in the reaction mixture by lipases from *Asp. niger* and *Rh. delemar*, while 1(3)-monoolein, 2-monoolein, 1,3-diolein and 1,2(2,3)-diolein were accumulated by lipases from *Geo. candidum* and *Pen. cyclopium*. *Geo.*

TABLE II. FATTY ACID COMPOSITION OF HYDROLYSIS PRODUCTS BY FOUR LIPASES

Hydrolysis products from cocoa butter were assayed by use of TLC and GLC. Experiment is described in detail in MATERIALS AND METHODS.

Product	Fatty acid	Mole % of fatty acid			
		<i>Asp.</i>	<i>Rh.</i>	<i>Geo.</i>	<i>Pen.</i>
1(3)-Mono-glyceride	Palmitic			21.5	35.9
	Stearic			63.0	46.1
	Oleic			14.3	17.9
	Linoleic			1.2	trace
2-Mono-glyceride	Palmitic	4.0	3.5	26.9	4.6
	Stearic	3.6	1.5	27.5	2.7
	Oleic	85.0	86.0	44.6	85.9
	Linoleic	6.6	8.0	1.0	6.5
1,2(2,3)-Diglyceride	Palmitic	18.7	20.2	17.2	20.0
	Stearic	26.0	26.7	32.3	23.9
	Oleic	51.3	49.9	49.3	52.3
	Linoleic	3.9	3.2	1.3	3.7
1,3-Di-glyceride	Palmitic			28.5	39.2
	Stearic			62.8	52.6
	Oleic			8.7	8.1
	Linoleic			trace	trace
Liberated fatty acid	Palmitic	41.8	37.0	36.3	30.3
	Stearic	50.6	53.2	9.6	46.4
	Oleic	7.7	8.0	48.2	22.3
	Linoleic	trace	0.1	5.9	1.0

candidum lipase accumulated a rather lower level of monoglyceride compared with the other three lipases at any stage of hydrolysis.

Hydrolysis of diolein mixture

Four lipases were allowed to react with the mixture of 1,3-diolein and 1,2(2,3)-diolein. TLC of the hydrolysis products is shown in Fig. 3. Each spot in the chromatogram was measured with a densitometer. Figure 4 shows the mode of the decrease of substrates. Either 1,3-diolein or 1,2(2,3)-diolein was hydrolyzed by lipases from *Geo. candidum* and *Pen. cyclopium*. Whereas, lipases from *Asp. niger* and *Rh. deleamar* hydrolyzed 1,3-diolein more rapidly than 1,2(2,3)-diolein.

Hydrolysis of cocoa butter

Cocoa butter was also employed to clarify the positional specificities of the lipases. Cocoa butter was hydrolyzed by each lipase to approximate 20% of the hydrolysis within

20 min. The fatty acid proportion of each product is shown in Table II.

The lipases from *Asp. niger* and *Rh. deleamar* gave almost the same results. The proportions of fatty acids liberated by these two lipases were found to be almost the same as those in position 1(3) of the original cocoa butter. Fatty acid proportions of 2-monoglyceride and those in position 2 of the original cocoa butter were the nearly same (Tables I and II).

On the other hand, in fatty acids liberated by lipases from *Geo. candidum* and *Pen. cyclopium*, the percentages of unsaturated fatty acids were fairly high. In liberated fatty acids by the action of *Geo. candidum* lipase, the percentage of stearic acid was remarkably low compared to oleic acid and palmitic acid. The fatty acid proportions of 1,3-diglyceride and 2-monoglyceride produced by *Pen. cyclopium* lipase corresponded closely to the fatty acid proportions in position 1(3) and in position 2 (Table I), respectively.

FIG. 4. Mode of Hydrolysis of Diolein Isomers by Four Lipases.

Experiment is described in detail in the text. —●—, 1,3-diolein; —○—, 1,2(2,3)-diolein.

DISCUSSION

With respect to hydrolyzates of triolein by action of lipases from *Asp. niger* and *Rh. deleamar*, 1,2(2,3)-diolein, 2-monoolein and free fatty acids were detected but not 1,3-diolein and 1(3)-monoolein (Fig. 2). The fatty acid proportions of liberated fatty acid and 2-monoglyceride from cocoa butter (Table II) agreed well with proportions in position 1(3) and in position 2 of the original cocoa butter (Table I), respectively. From these facts, it is clear that lipases from *Asp. niger* and *Rh. deleamar* do not hydrolyze the ester bond in position 2 of triglyceride and are nonspecific for fatty acids constituting cocoa butter. These two lipases hydrolyzed 1,3-diolein in preference to 1,2(2,3)-diolein when they were present to-

FIG. 5. The Principal Reaction Sequences of the Lipases.

a) Lipases from *Asp. niger* and *Rh. deleamar*.

b) Lipases from *Geo. candidum* and *Pen. cyclopium*.

[—], glycerol; R, fatty acid; $\updownarrow$, spontaneous isomerization; $\longrightarrow$, easily hydrolyzed; $\longrightarrow$, hydrolyzed; $\cdots$, not hydrolyzed; $\square$, accumulated in the reaction mixture.

gether in reaction mixture (Fig. 4). Figure 5-a shows the possible reaction sequences during the hydrolysis of triglyceride by lipases from *Asp. niger* and *Rh. deleamar*. Judging from their positional specificities, these two lipases may be applied to study the positional distribution of fatty acids in triglyceride in a manner similar to pancreatic lipase.

On the other hand, lipases from *Geo. candidum* and *Pen. cyclopium* hydrolyzed triolein and accumulated 1,2(2,3)-diolein, 1,3-diolein, 1(3)-monoolein and 2-monoolein in the reaction mixture (Fig. 2). They also hydrolyzed 1,3-diolein and 1,2(2,3)-diolein (Fig. 4). The percentages of unsaturated fatty acids liberated from cocoa butter by these two lipases were remarkably higher than those in position 1(3) of the original cocoa butter (Tables I and II). These facts suggest that lipases from *Geo. candidum* and *Pen. cyclopium* hydrolyze all ester bonds of triglyceride regardless of their positions.

The fatty acid proportions of 2-monoglyceride and 1,3-diglyceride produced by *Pen. cyclopium* lipase agreed well with those in position 2 and in position 1(3) of the original

cocoa butter, respectively. From these facts, it is assumed that *Pen. cyclopium* lipase liberates all kinds of fatty acids at each position in cocoa butter triglyceride with the same velocity. However, the percentage of stearic acid in fatty acids liberated by *Geo. candidum* lipase was remarkably low compared with that liberated by the other three lipases (Table II). It is clear that *Geo. candidum* lipase liberates oleic acid and palmitic acid in preference to stearic acid from cocoa butter. Alford *et al.*⁹⁾ and Sampugna and Jensen¹⁸⁾ have shown that lipase from *Geo. candidum* preferentially liberates unsaturated fatty acids regardless of their positions in triglyceride. In our study, as indicated above, lipase from *Geo. candidum* Link easily liberated not only oleic acid but palmitic acid. These two microorganisms named *Geo. candidum* are different strains as previously reported.¹⁹⁾ Therefore, differences in substrate specificities between these two *Geo. candidum* lipases may be possible. The assumed reaction sequences of lipases from *Geo. candidum* and *Pen. cyclopium* are shown in Fig. 5-b.

Triolein was detected on TLC of hydrolysis products from diolein (Fig. 3). It was previously demonstrated to be formed mainly by the esterifying action of lipases under the experimental condition.²⁰⁾ Detailed studies on the formation of triglyceride by the lipases will be published elsewhere.

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