Lipase-Catalyzed Reactions Involving Thia Fatty Acids and Ester Derivatives

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ABSTRACT: Enzymatic hydrolysis of synthetic methyl 5-, 9-, and 12-thiastearates in aqueous media with *Candida cyclindracea* or porcine pancreatic lipases gave the corresponding fatty acids in 70–100% yield. Hydrolysis of the 3- and 4-positional isomers gave only 15–25% of the free thia fatty acids, suggesting discrimination against these isomers by lipases. No lipolysis was achieved with methyl 2-thialaurate under a range of reaction conditions. Esterification of the 3-, 4-, 5-, 9-, and 12-thiastearic acids with *n*-butanol in *n*-hexane using Lipozyme (immobilized *Rhizomucor miehei*) as the biocatalyst gave the corresponding butyl esters in 80–95% yield. Interesterification (acyl exchange) of triolein with methyl 9-thiastearate in the presence of Lipozyme showed the incorporation of 9-thiastearoyl chain at only one of the α-positions of triolein. In the case of methyl 2-thialaurate, no lipase-catalyzed acyl exchange reaction was possible. This study showed that the position of the sulfur atom in thia fatty esters affects the lipase-catalyzed hydrolysis and interesterification reactions.

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KEY WORDS: Esterification, hydrolysis, interesterification, lipase, thia fatty acids.

Synthetic long-chain fatty acids with a methylene group "replaced" by a sulfur atom (thia fatty acids) have been used in biological studies. These fatty acid analogues possess anticholesteremic, hypolipemic, and antimicrobial properties (1–3) and inhibit leukotriene biosynthesis (4). Triacylglycerols (TAG) are of special interest in the metabolism and absorption of fats in humans and other mammals (5). Several *in vivo* biological studies with TAG containing active fatty acids such as docosahexaenoic acid, eicosapentaenoic acid, and γ-linolenic acid have been reported (6–9). It is essential to know the activity of lipases toward particular fatty acids under study for *in vivo* absorption and metabolism studies. We therefore report the activity of lipases on the hydrolysis of several positional isomers of thia fatty esters, the esterification of such thia fatty acids, and the interesterification of thiastearates with triolein.

MATERIALS AND METHODS

Thia fatty acids (*viz.*, 3-, 4-, 5-, 9-, and 12-thiastearic acids and methyl 2-thialaurate) were synthesized according to procedures described elsewhere (10). Triolein was synthesized by lipase (Novozyme 435) catalyzed esterification of glycerol with oleic acid (11). Lipolase 100T (activity, 100 KLU/g), Lipozyme IM20 (activity, 25 BIU/g—two forms of lipases from the fungus *Rhizomucor miehei*) and Novozyme 435 (*Candida antarctica*, activity 40 BIU/g) were gifts from Novo Nordisk A.S. (Hong Kong). One KLU (Kilo lipase unit) corresponds to 1 μmol butyric acid formed from tributyrin per min, and 1 BIU (batch interesterification unit) corresponds to 1 μmol of palmitic acid incorporated into triolein per min under the standard conditions as defined by the manufacturer. *Candida cylindracea* (CCL) (type VII, activity 905 U/mg, 1 Unit will hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in 1 h at pH 7.2 at 37°C using olive oil) and porcine pancreatic lipases (type II, 179 U/mg, 1 Unit will hydrolyze 1.0 microequivalent of fatty acid from a triglyceride in 1 h at pH 7.7 at 37°C using triolein) were purchased from Sigma Chemical Co. (St. Louis, MO). Thin-layer (TLC), column, gas–liquid chromatographic (GLC), infrared (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopic analyses were performed as described elsewhere (12).

Lipase-catalyzed hydrolysis of thia fatty acid methyl esters as exemplified by the hydrolysis of methyl 9-thiastearate with CCL lipase. A mixture of methyl 9-thiastearate (0.1 g), *n*-hexane, CCL lipase powder (20 mg), and potassium phosphate buffer (2 mL, 0.1 M, pH 7.1) was stirred in a screw-capped test tube at 40°C for 24 h. Diethyl ether (2 mL) was added and centrifuged. The ethereal phase was separated and the aqueous phase extracted with diethyl ether (2 × 2 mL). The ether extracts were combined and evaporated under a stream of nitrogen. An azeotropic mixture consisting of benzene/chloroform/methanol (1:1:1, vol/vol/vol), 1 mL, was added. The solvent was evaporated to give a concentrate of 9-thiastearic acid (0.9 g). Likewise, the hydrolysis of 3-, 4-, 5-, and 12-thia esters with CCL lipase was carried out.

Lipase-catalyzed esterification of thia fatty acids as exemplified by the esterification of 9-thiastearic acid with n-*butanol.* A mixture of 9-thiastearic acid (0.2 g, 0.7 mmol), *n*-butanol (0.12 mL), *n*-hexane (1.7 mL), and Lipozyme (30 mg) was stirred in a glass culture tube at 60°C for 2 h. The reaction mixture was centrifuged, and *n*-hexane solution was loaded onto a short silica gel (2 g) column (250 mm × 10 mm i.d.) and eluted with a mixture of *n*-hexane and diethyl ether (4:1, vol/vol, 25 mL). Evaporation of solvents under reduced

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pressure gave pure butyl 9-thiastearate (0.24 g, 96%). In a similar way, 3-, 4-, and 5-thia stearic acids were esterified to their butyl esters in good yield in each case.

Lipase-catalyzed interesterification of thia fatty esters as exemplified by the incorporation of 9-thiastearoyl chain into triolein. A mixture of triolein (0.2 g, 0.23 mmol), methyl 9-thiastearate (0.14 g, 0.45 mmol), Lipozyme (34 mg), and *n*-hexane (2 mL) was stirred at 60°C for 4 h. The mixture was centrifuged, and the supernatant liquid was eluted through a silica column (10 g) using 25 mL of petroleum ether (b.p. 40–60°C) and diethyl ether (4:1, vol/vol) to give 1-(9-thia)stearoyl-2,3-dioleoylglycerol (0.19 g, 91%) as a colorless oil. The R_f was 0.80 in 5% diethyl ether in petroleum ether.

^{1}H-NMR ($CDCL_3$, δ): 0.88 (*t*, 9H, CH_3), 1.20–1.50 (*m*, 50H, CH_2), 1.50–1.70 (*m*, 18H, CH_2), 2.01 (m, 8H, CH_2CH=CH), 2.30 (*t*, 4H, oleoyl CH_2COO), 2.33 (2H, thiastearoyl CH_2COO), 2.50 (*t*, 4H, CH_2-S-CH_2), 4.1–4.35 (*m*, 4H, glyceryl CH_2), 5.2–5.4 (*m*, 5H, glyceryl CH + olefinic CH). ^{13}C-NMR ($CDCl_3$, ppm): 14.13 (C-18, 3C), 22.71 (C-17, 3C), 24.86 (C-3, 2C, α-acyl), 24.92 (C-3, β-acyl), 27.18 (C-8, 2C, oleoyl), 27.23 (C-11, 2C, oleoyl), 28.76, 28.91, 28.99, 29.03, 29.10, 29.14, 29.20, 29.29, 29.34, 29.52, 29.55, 29.66, 29.75, 29.78, 31.92 (C-16), 32.13 (C-8, thiastearoyl), 32.21 (C-10, thiastearoyl), 34.03 (C-2, α-oleoyl), 34.20 (C-2, β-oleoyl), 34.33 (C-2, α-thiastearoyl), 62.11 (glyceryl CH_2), 68.88 (glyceryl CH), 129.68 (C-9, α-oleoyl), 129.71 (C-9, β-oleoyl), 129.76 (C-10, α-oleoyl), 130.02 (C-10, β-oleoyl), 172.85 (C-1, β-oleoyl), 173.26 (C-1, α-oleoyl) and 173.82 (C-1, α-thiastearoyl); IR (NaCl, cm^{-1}): 3000 (m) and 1740 (C=O ester).

A similar interesterification reaction was carried out using methyl 2-thialaurate with triolein. No incorporation of any 2-thialauroyl chain into triolein was observed.

RESULTS AND DISCUSSION

The various lipase-catalyzed reactions involving thia fatty acids and esters are presented in Figure 1. The amount of fatty acids formed upon CCL lipase-catalyzed hydrolysis of positional isomers of methyl thia fatty esters (*viz.*, methyl 3-, 4-, 5-, 9-, and 12-thia stearates and methyl 2-thialaurate) in potassium phosphate buffer (pH 7.0, 0.1 M) is 15, 25, 70, 86, and 100%, respectively. The CCL lipase-catalyzed hydrolysis of methyl 9-thiastearate in phosphate buffer at 40°C (24 h) gave 9-thiastearic acid in 86% yield. The structure of 9-thiastearic acid was confirmed by ^{1}H and ^{13}C NMR spectroscopic techniques (13). Replacing CCL with porcine pancreatic lipase (PPL) in *tris*-buffer gave similar results, but the rate of hydrolysis was comparatively lower.

When the hydrolysis reaction was performed on methyl 2-thialaurate, no corresponding fatty acid could be isolated. Methyl 2-thialaurate was not acted upon by a range of lipases (viz. CCL, PPL, Lipozyme, Lipolase). Furthermore, hydrolysis of methyl 2-thialaurate could not be induced by increasing the amount of lipases (to 5-fold), incremental increases in the

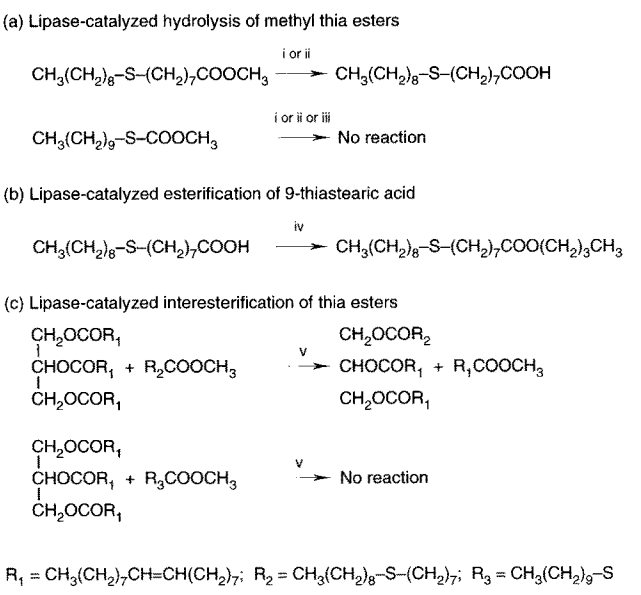

FIG. 1. The various lipase-catalyzed reactions involving thia fatty acids and esters. Reagents: i, *Candida cylindracea* lipase, potassium phosphate buffer, 40°C, 24 h; ii, porcine pancreatic lipase, *tris*-buffer, 40°C, 24 h; iii, Lipolase, potassium phosphate buffer, 50°C, 7 d; iv, $CH_3(CH_2)_3$ OH, Lipozyme, *n*-hexane, 60°C, 2 h; v, Lipozyme, *n*-hexane, 60°C, 4 h. Lipolase and Lipozyme from Novo Nordisk A.S. (Hong Kong).

reaction temperature from 25 to 60°C, or by prolonging the reaction time to 7 d at 25°C. To ensure that the lipases were not deactivated by methyl 2-thialaurate, a mixture of methyl oleate and methyl 2-thialaurate was stirred in phosphate buffer in the presence of CCL at 25°C for 2 h. Separation of the resulting product by silica column chromatography showed that the free fatty acid fraction consisted of oleic acid only, while the methyl ester fraction contained exclusively the unreacted methyl 2-thialaurate.

Similar reactions were repeated using different positional isomers of thia fatty esters. The 3- and 4-thia fatty ester isomers are not readily hydrolyzed by CCL. In the case of 5-thiastearate, hydrolysis within the same timeframe gave only 70% of the corresponding thia fatty acid. With the sulfur atom located at remote distances from the methyl ester group (9- and 12-position), hydrolysis of the ester function appeared to be highly successful with yields reaching 86 and 100%, respectively.

It is known that lipases in hydrolysis reactions of esters are sensitive to steric effects on the acid side rather than the alcohol side (14). Heimermann *et al.* (15) have reported that the C_{18} monounsaturated fatty acids from TAG with the double bond at the 2- to 7-position were resistant to pancreatic lipase hydrolysis. Miller *et al.* (16) have indicated that an unsaturated carboxylic acid containing a double bond at the 2-position (e.g., cinnamic acid) did not undergo Lipozyme-catalyzed esterification. Our result involving the 2-thia ester showed a similar behavior during lipolysis. The lone pairs of electrons of the sulfur atom located adjacent to the carbomethoxy group present a similar electron-rich situation as

that in 2-ethylenic fatty acids. Such effects appear to retard the hydrolysis of the ester group in methyl 2-thialaurate. Therefore, the effect exerted by the lone pairs of electrons of the sulfur atom from the 2-position seems to prevent the formation of enzyme-substrate complex.

The reactivity of thia fatty acids upon lipase-catalyzed esterification with *n*-butanol was also investigated using Lipozyme (immobilized *R. miehei* lipase). Lipozyme is known for its efficiency in catalyzing esterification reactions. Reaction of 9-thiastearic acid with *n*-butanol in *n*-hexane in the presence of Lipozyme at 60°C for 2 h gave butyl 9-thiastearate in excellent yield (>95%). Extension of the reaction to the 3-, 4-, 5-, and 6-thia isomers also afforded the corresponding butyl esters in high yields (85–95%). Use of CCL for the same esterification reaction with *n*-butanol in *n*-hexane at 40°C was rather sluggish (24 h) and gave the butyl esters in 5–20% yield. As free 2-thialauric acid could not be prepared due to its labile nature (13), the esterification reaction of 2-thia fatty acid with *n*-butanol was therefore not investigated.

From the lipase-catalyzed hydrolysis of methyl thia fatty esters, we have shown that the sulfur atom, when located at the 9-position or beyond, does not affect the rate of hydrolysis. We have therefore chosen methyl 9-thiastearate to test the incorporation of a thia acyl chain into triolein.

In interesterification (acyl exchange) studies, Lipozyme is known for its 1,3-positional specificity (17,18). Our earlier studies showed that optimal incorporation of a synthetic furanoid fatty acyl chain at one of the α-positions may be achieved with a mixture of triolein and methyl ester in a molar ratio of 1:2 in *n*-hexane at 60°C for 4 h (12). Under the same conditions, reaction of methyl 9-thiastearate with triolein in a molar ratio of 2:1 in the presence of Lipozyme in *n*-hexane gave 1-(9-thiastearoyl)-2,3-dioleoylglycerol in 91% yield. Gas chromatographic analysis of the methyl esters derived from the hydrolyzed product by reaction with trimethylsulfonium hydroxide showed the presence of 9-thiastearic and oleic acids in a 1:2 ratio. The incorporation of a single thiastearoyl chain into triolein was evident from the structural analysis. From the ^{1}H NMR spectrum (13), the signal at δ 2.50 (*t*, *J* 7.0 Hz) with an integrated intensity equivalent to four protons showed the presence of the 9-thiastearoyl chain. The signal at δ5.34 associated with the olefinic system of the derived compound corresponded to four protons. From these spectral results, it was apparent that only one thiastearoyl chain was present in the sample. This observation was confirmed by ^{13}C NMR analysis (13). The presence of α- and β-oleoyl chains in the derived compound was confirmed by the appearance of two pairs of signals (of about equal intensities) for the olefinic carbon atoms: *viz.*, 129.68/129.71 and 129.776/130.030 ppm, which corresponded to the shifts of C-9 and C-10 in the α- and β-actyl positions, respectively (12). The carbon shifts of the methylene carbon atoms adjacent to the sulfur atom in the 9-thiastearoyl chain appeared at δ32.13 (C-8) and 32.21 (C-10) ppm (13).

In order to ascertain whether there was any incorporation of the 9-thiastearoyl chain during the lipase-catalyzed interesterification process into the β-position of triolein, PPL (a 1,3-regiospecific lipase) catalyzed hydrolysis was carried out on 1-(9-thia)stearoyl-2,3-dioleoylglycerol according to the procedure described by Luddy *et al.* (19). The lipolysis products were extracted, and the 2-monoacylglycerol (2-MAG) fraction was separated by TLC. The 2-MAG was transesterified with trimethylsulfonium hydroxide to give methyl esters. GLC analysis identified the methyl ester as oleate. This analysis demonstrated that the β-oleoyl chain in triolein remained intact during the interesterification reaction, and the 9-thiastearoyl chain was therefore regiospecifically incorporated by Lipozyme at one of the α-positions of triolein. The PPL-catalyzed hydrolysis of 1-(9-thia)stearoyl-2,3-dioleoylglycerol also showed that the 9-thiastearoyl group in the α-acyl position was not discriminated against during the lipolysis. When methyl 2-thialaurate was used as the substrate instead of methyl 9-thiastearate in the interesterification reaction under the same conditions, no incorporation was observed and the starting materials were recovered.

We conclude from this study that the enzymatic hydrolysis and interesterification of synthetic thia fatty esters containing the sulfur atom at the 5-position or beyond from the ester group can be readily achieved. Thia fatty acids, including the 3- and 4-positional isomers, are successfully esterified to *n*-alkyl esters. However, methyl 2-thialaurate resists all lipase-catalyzed reactions investigated in this study due to the effects exerted by the lone pairs of electrons of the sulfur atom at the 2-position.

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