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STEROLS AND FATTY ACIDS FROM THREE SPECIES OF MANGROVE

SUNITI MISRA, A. CHOUDHURY, ASHIS K. DUTTA* and AMITABHA GHOSH*†

Marine and Estuarine Biological Research Unit, Department of Marine Science, University of Calcutta, 35 B.C. Road, Calcutta 700019, India; *Department of Chemistry, Bose Institute, 93/1 A.P.C. Road, Calcutta 700009, India

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Key Word Index—*Acanthus ilicifolius*; Acanthaceae; *Bruguiera gymnorhiza*; Rhizophoraceae; *Rhizophora mucronata*; Rhizophoraceae; mangrove; lipolysis; steryl ester; sterol; fatty acid.

Abstract—The hydrolysis of steryl esters on thin-layer chromatographic plates by porcine pancreatic lipase is described. The sterols and fatty acids produced were separated on the same plate, recovered, and analysed by gas–liquid chromatography for their compositions. Synthetic cholesteryl esters containing various saturated and unsaturated fatty acids and synthetic steryl oleates with various sterols were lipolysed along with steryl esters of *Acanthus ilicifolius*, *Bruguiera gymnorhiza* and *Rhizophora mucronata* mangrove leaves. The major sterol was sitosterol which was accompanied by cholesterol, campesterol, stigmasterol and 28-isofucosterol. In addition, stigmast-7-en-3β-ol was present in *R. mucronata* leaves. The component fatty acids found in all three species were 16:0, 18:0, 18:1, 18:2 and 18:3. The relative proportions of the sterols and fatty acids were significantly different from the chemotaxonomic standpoint. The results obtained by carrying out plate lipolysis for 45 min at 40° compared well with those produced by conventional chemical hydrolysis.

INTRODUCTION

Porcine pancreatic lipase is known to possess various carboxyl esterase activities [1, 2] although it is used extensively for deacylation of triglycerides for compositional studies [3–6]. The steryl esterase activity of pancreatic lipase has been investigated by various workers [7–9] and considerable work has been done on several aspects [10–13] of the enzyme responsible for this activity. In a previous study by Misra *et al.* [14], the wax ester cleaving activity of porcine pancreatic lipase was used on thin-layer chromatographic plates, for compositional studies of natural and synthetic wax esters. In the present paper, the steryl esterase activity of porcine pancreatic lipase has been utilized to hydrolyse mangrove leaf steryl esters on thin-layer chromatographic plates and the compositions of the fatty acids and sterols have been determined.

RESULTS

Effect of temperature on the extent of lipolysis

The effect of incubation temperature on the extent of lipolysis was studied using cholesteryl oleate at various temperatures between 20° and 50°, and the results are shown in Fig. 1. The results show that, for 30 min incubations at various temperatures, the extent of hydrolysis increased gradually and reached a maximum at 40°.

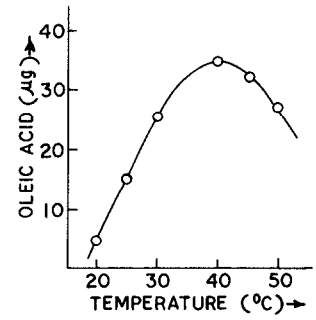

Fig. 1. Effect of temperature on the lipolysis of cholesteryl oleate. On each plate, 1 mg of cholesteryl oleate was incubated with 2 mg of lipase and 2 mg of cholic acid in veronal buffer (pH 8.6, 0.1 M) at various temperatures, for 30 min each.

Time-course study of the lipolysis

The time course of the on-plate lipolysis of cholesteryl oleate is presented in Fig. 2. The extent of lipolysis at

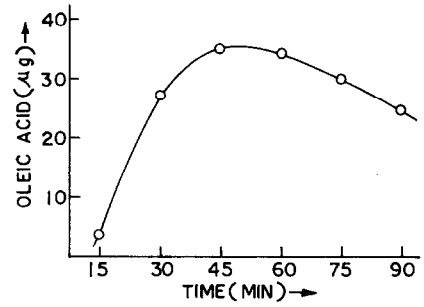

Fig. 2. Time course of on-plate lipolysis of cholesteryl oleate. On each plate, 1 mg of cholesteryl oleate was incubated with 2 mg of lipase and 2 mg of cholic acid in veronal buffer for various time periods at 40° each.

†To whom correspondence should be addressed.

various times between 15 and 90 min was studied, using an incubation temperature of 40°. The results show that the extent of hydrolysis increased sharply to a maximum at 45 min.

Effect of enzyme concentration on the extent of hydrolysis

Various amounts of enzyme between 1 and 16 mg were each incubated with 1 mg of cholesteryl oleate at 40° for 45 min. The results are shown in Fig. 3. The extent of lipolysis gradually increased and became constant with 12 mg of the enzyme.

Effect of cholic acid concentration on the extent of hydrolysis

Various amounts of cholic acid between 2 and 12 mg were each incubated with 1 mg of cholesteryl oleate and 12 mg of enzyme, at 40° for 45 min. The results presented in Fig. 4 indicate that the extent of hydrolysis gradually increased and became constant with 10 mg of cholic acid.

Optimum conditions for lipolysis of steryl esters on TLC plates

The best result was obtained by incubating 1.0 mg of cholesteryl oleate with 12.0 mg of enzyme and 10.0 mg of cholic acid in 0.5 ml of veronal buffer (pH 8.6, 0.1 M) for 45 min at 40°, on a TLC plate with a 0.25 mm thick silica gel layer. Under these conditions the extent of lipolysis of cholesteryl oleate was 64 %, as determined from the data in Fig. 4.

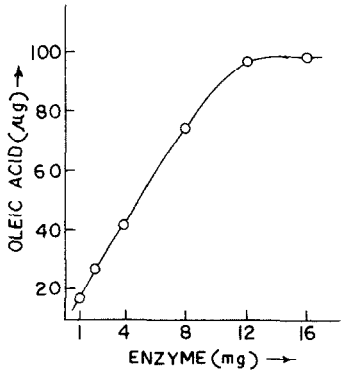

Fig. 3. Effect of enzyme concentration on the extent of lipolysis of cholesteryl oleate. On each plate, 1 mg of cholesteryl oleate was incubated with 2 mg of cholic acid and various amounts of lipase in veronal buffer. Each plate was incubated for 45 min at 40°.

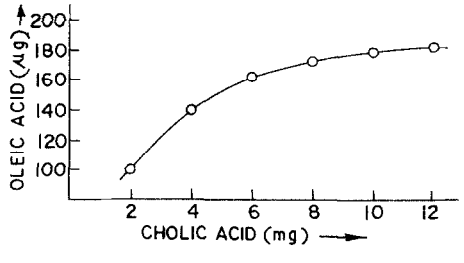

Fig. 4. Effect of cholic acid concentration on the extent of lipolysis. On each plate, 1 mg of cholesteryl oleate was incubated with 12 mg of enzyme and various amounts of cholic acid. Incubation was for 45 min at 40°.

Lipolysis of synthetic and leaf steryl esters

The synthetic and leaf steryl esters (1 mg each) were lipolysed on TLC plates using the optimum conditions stated earlier. The compositions of the fatty acids of the synthetic and leaf steryl esters, obtained by lipolysis and chemical hydrolysis, as determined by GLC are compared in Table 1. Sterol compositions of the samples are presented in Table 2.

Double-bond positions of the unsaturated acids

Permanganate–periodate oxidation of the monoenes and those obtained by the partial reduction of the dienes and trienes revealed the presence of C_9-, C_{12}- and C_{15}-dicarboxylic acids by GLC. Catalytic reduction of the unsaturated acids yielded stearic acid. These facts confirmed that mono-, di- and trienes had double bonds at the Δ^9, $\Delta^{9,12}$ and $\Delta^{9,12,15}$-positions, respectively.

DISCUSSION

The presence of an enzyme in pancreatic juice and extracts which hydrolyses long-chain fatty acid esters of cholesterol was demonstrated by Mueller [15, 16]. It was given the name steryl ester hydrolase (EC 3.1.1.13) by the Enzyme Commission. It was demonstrated [8, 17] that cholesteryl esterase of the pancreas and small intestine possesses hydrolytic and synthetic activities. Murthy and Ganguly [9] have studied in detail various aspects of the hydrolytic and synthetic activities of the enzyme from rat pancreas and intestine. Recently, steryl ester hydrolase activity was demonstrated [18] in a higher plant.

Pancreatic lipase has been extensively used for hydrolysing triglycerides [3–6] and it is also known to hydrolyse the acyl ester linkages of phospholipids [19, 20], galactolipids [21] and wax esters [3]. The present study was undertaken to make use of the steryl esterase activity of pancreatic lipase in the analysis of natural steryl esters.

The effect of incubation temperature, as presented in Fig. 1, indicates that the optimum temperature for lipolysis on the TLC plate was 40°, whereas Murthy and Ganguly [9] used 37°. The time course of the reaction, as shown in Fig. 2, indicated that the optimum time for on-plate lipolysis was 45 min.

The effect of the enzyme concentration study indicated (Fig. 3) that an enzyme to substrate ratio of 12:1 is ideal for on-plate lipolysis. The high enzyme concentration required for steryl esterase activity may be due to the low concentration of this enzyme in the commercial lipase preparation, which is normally used for lipolysis of triglycerides.

It was shown by Murthy and Ganguly [9] that hydrolytic activity increases sharply compared to synthetic activity in the presence of various bile salts and it is highest in the presence of cholic acid. In the present study, cholic acid was chosen and its effect, after study at various concentrations (Fig. 4), indicated that, for 1 mg of substrate and 12 mg of enzyme, 10 mg of cholic acid is needed under specified conditions, for the highest hydrolytic activity (Fig. 4).

Table 1. Fatty acid compositions (% w/w) of leaf steryl esters and synthetic cholesteryl esters obtained by chemical and lipase hydrolysis

Fatty acids*	*A. ilicifolius*		*B. gymnorhiza*		*R. mucronata*		Synthetic	
	L†	C†	L	C	L	C	L	C
16:0	15.0	15.7	26.4	25.9	41.8	42.4	45.0	43.2
18:0	6.9	6.4	5.3	5.4	1.9	2.0	26.0	28.0
18:1(9)	23.5	23.0	15.4	14.9	3.8	4.0	11.0	11.6
18:2(9)	32.1	31.8	33.1	33.3	29.8	28.4	13.0	12.3
18:3(9)	22.5	23.1	19.8	20.5	22.7	23.2	5.0	4.9

*Double-bond positions are numbered from the carboxyl group, shown in parentheses. All subsequent double bonds are methylene-interrupted.

†L = Obtained by lipase hydrolysis; C = obtained by chemical hydrolysis.

Table 2. Sterol compositions (% w/w) of leaf steryl esters and synthetic steryl oleates obtained by chemical and lipase hydrolysis

Sterols	*A. ilicifolius*		*B. gymnorhiza*		*R. mucronata*		Synthetic	
	L*	C*	L	C	L	C	L	C
Cholesterol	5.3	5.7	6.0	6.6	2.7	2.7	20.5	21.0
Campesterol	16.0	16.9	8.3	8.6	14.2	14.0	21.6	22.0
Stigmasterol	13.5	13.2	7.5	7.6	8.0	8.5	28.5	27.0
Sitosterol	54.5	54.0	56.8	56.2	60.0	58.8	29.4	30.0
28-Isofucosterol	10.7	10.2	10.5	9.5	5.0	5.2	—	—
Stigmast-7-en-3β-ol	—	—	—	—	10.1	10.8	—	—
Unidentified	—	—	10.9	11.5	—	—	—	—

*L = Obtained by lipase hydrolysis; C = obtained by chemical hydrolysis.

The effect of pH on the hydrolytic and synthetic activities was investigated by Murthy and Ganguly [9], who observed that, for cholesteryl oleate, the optimum pH was 8.6 for the pancreatic enzyme. In the present study, a pH of 8.6 was used throughout.

The results of the specificity study regarding sterols and fatty acids indicated that there was no compositional change in either of the compounds and that the enzyme hydrolysed the steryl esters randomly, irrespective of the incubation time. This fact confirms the conclusions made by Brockerhoff and Jensen [2] that neither sterol nor fatty acid is recognized by the enzyme.

The fatty acid compositions of the steryl esters of the three samples of leaves and that of the synthetic cholesteryl ester (Table 1) obtained by chemical and lipase hydrolysis indicate that there is good agreement between the results. Table 1 indicates that in all the three plant species studied, linoleic (18:2) acid is the major component. Among the other unsaturated constituents, oleic (18:1) and linolenic (18:3) acids were present. Of the saturated acids, palmitic (16:0) acid was the major component in all the samples, the other component being stearic (18:0) acid. The fatty acid compositions of the total lipids of mangrove, as reported earlier [22, 23], indicated that the major constituents were 16:0, 18:0, 18:1 (9), 18:2 (9, 12) and 18:3 (9, 12, 15). A similar fatty acid composition of the steryl esters was found in the present study. It is interesting to note that the chain lengths of the fatty acids of the steryl esters were restricted to only C_{16} and C_{18}, whereas the chain lengths in the total lipid may vary from C_{12} to C_{24} [22, 23]. Significant quantitative differences in the fatty acid make-up were observed between the three species; this is chemotaxonomically important.

The sterol compositions of leaf steryl esters and those of synthetic steryl oleates, as presented in Table 2, indicate that there is good agreement between the sterol compositions obtained by lipase and chemical hydrolysis. The sterol compositions of the plant leaf steryl esters studied indicate that the major sterol was sitosterol in all the samples and this constituted over 50% of the total sterols, as expected for many higher plants [24]. Cholesterol was highest in *B. gymnorhiza*, whereas campesterol and stigmasterol were highest in *A. ilicifolius*, as reported earlier [23]. The concentration of 28-isofucosterol was lowest in *R. mucronata*, which also contained stigmast-7-en-3β-ol, which was absent in the other species. An unidentified component was present in *B. gymnorhiza*. The sterol compositions indicate that chemotaxonomically significant differences exist between the species.

The moisture content (Table 3) of the leaves varied between 55 and 66%. Total lipid was highest in *B. gymnorhiza*, whereas steryl ester was highest in *R. mucronata*.

A comparison of the fatty acid and sterol compositions (Tables 1 and 2) of the leaf steryl esters by chemical and lipase hydrolysis indicated that there is good agreement between the results and that lipolysis of steryl esters on TLC plates can be used for compositional studies using a commercial lipase preparation.

EXPERIMENTAL

Samples. Leaf samples were collected from Prentice Island, between latitudes 21.43° and 21.46° N and longitudes 88.18° and

Table 3. Percentages of moisture, total lipid, and steryl ester of three species of mangrove leaves

Species	Moisture %	Total lipid*	Steryl ester*
A. ilicifolius	60.0	1.5	0.05
B. gymnorhiza	66.0	4.0	0.10
R. mucronata	55.0	2.0	0.40

*Per cent of dry leaf.

88.19° E of the Sunderban mangrove forest, West Bengal, India. The species studied were *Acanthus ilicifolius*, *Bruguiera gymnorhiza* and *Rhizophora mucronata*.

Lipid extraction. Leaves were washed, cut into pieces, and homogenized with MeOH–$CHCl_3$ (2:1), centrifuged and the residue was again homogenized with MeOH–$CHCl_3$–H_2O (2:1:0.8). After centrifugation, the residue was again homogenized with MeOH–$CHCl_3$ (2:1) and recentrifuged. The supernatants were pooled and diluted with $CHCl_3$–H_2O (1:1) as in ref. [25]. The lower $CHCl_3$ layer was dried over dry Na_2SO_4.

Isolation of leaf steryl esters. Prep. TLC of the lipids was carried out as described in ref. [26].

Synthesis of steryl esters. Individual steryl oleates and cholesteryl esters containing various fatty acids were prepared by condensing various sterols with various acyl chlorides as described elsewhere [14]. The structures of the steryl esters were confirmed by IR and MS.

Preparation of lipase solution. Hog pancreatic lipase (type II, Sigma Chemical Co., St. Louis, MO, U.S.A.) was washed free of lipid contaminants using Et_2O (6×) and Me_2CO (6×) as described in ref. [27]. A saturated soln of lipase in 0.1 M veronal buffer [9], pH 8.6, was prepared, centrifuged and the supernatant was used. The soluble protein concn of this soln was determined [28]. The amount of soluble protein was referred to as the amount of lipase. The activity of this lipase soln was found [6] to be 100 units per mg of protein, using olive oil as substrate. The activity of this lipase soln was 0.06 unit per mg of protein, using cholesteryl oleate as substrate. The unit activity is defined as the amount that will hydrolyse 1 μmol of oleic acid from cholesteryl oleate in 1 hr at pH 8.6 and 37°.

Determination of the optimum conditions for lipolysis on TLC plates. All the experiments were performed by following the general procedures described earlier [14]. In all the experiments, 1 mg cholesteryl oleate per 0.2 ml hexane was used. Enzyme soln in veronal buffer, pH 8.6, was used throughout. Optimum value parameters were determined as follows. For temperature, 1 mg substrate, 2 mg lipase and 2 mg cholic acid in 0.5 ml buffer were applied onto various TLC plates and incubated for 30 min each, between 20° and 50°. For time, the same procedure as in the above experiment was followed. Incubations were for various times between 15 and 90 min, all at 40°. For the enzyme to substrate ratio, various amounts of enzyme between 1 and 16 mg, each in 0.5 ml buffer containing 2 mg cholic acid together with 1 mg substrate, were incubated at 40° for 45 min. For cholic acid, the conditions were the same as in the enzyme to substrate ratio experiment, with various amounts of cholic acid between 2 and 12 mg and 12 mg enzyme in each case. After incubation in all the above experiments, each plate was placed into an Et_2O chamber in order to extract the products from the reaction zone [14]. After extraction, the plates were developed in a solvent consisting of petrol (40°–60°)–Et_2O–HOAc (80:20:1.5). Oleic acid was recovered, methylated [29] and GLC analysis was performed after adding 50 μg methyl pentadecanoate (C_{15}) to each of the samples for quantification [14].

Lipolysis of synthetic and leaf steryl esters on TLC plates. The synthetic and leaf steryl esters (1 mg) were incubated on TLC plates along with 12 mg lipase and 10 mg cholic acid in 0.5 ml veronal buffer, for 45 min at 40°. Sterols and fatty acids were separated and recovered from the plates, derivatized, and analysed by GLC.

Determination of sterol and/or fatty acid specificity. Synthetic cholesteryl esters with various fatty acids, and steryl oleates with various sterols were incubated and separated on TLC plates as described, for various incubation times from 15 to 90 min. Sterols and fatty acids were recovered, derivatized and analysed by GLC.

Chemical hydrolysis of synthetic and leaf steryl esters. The synthetic and leaf steryl esters (1 mg) were refluxed with 20 ml 0.01 M KOH in MeOH for 2 hr, MeOH was evapd, the mixture was diluted with H_2O and the sterols were extracted with Et_2O. The aq. phase was acidified with 2 M H_2SO_4 and the fatty acids were extracted with Et_2O. Sterols and fatty acids were derivatized and analysed by GLC.

GLC of fatty acids and sterols. GLC of fatty acids was performed using the methyl esters [14]. Acetyl derivatives [30] of the sterols were analysed on a 3% SE-30 column [31]. Trimethylsilyl derivatives [32] of sterols were analysed on a 3% OV-17 column [31]. Sterols were identified by comparing the RR_t of steryl acetates [33], retention indices [34] of sterol–OTMS derivatives [35] and the R_t with those of authentic standards.

Determination of double-bond positions of the unsaturated fatty acids. Fatty acids of leaf steryl esters were isolated by argentation CC [36]. Partial reduction with N_2H_4 and $KMnO_4$–KIO_4 oxidation were performed as described earlier [37].

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