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ENZYMATIC REACTIONS ON THIN-LAYER CHROMATOGRAPHIC PLATES

IV*. LIPOLYSIS OF WAX ESTERS AND SEPARATION OF PRODUCTS ON A SINGLE PLATE

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SUMMARY

Hydrolysis of wax esters on thin-layer chromatographic plates by porcine pancreatic lipase is described. The alcohols and free fatty acids produced were separated on the same plate, recovered and then analysed by gas–liquid chromatography for their compositions. Synthetic wax containing saturated fatty acids of various chain lengths and unsaturated fatty acids with varying numbers of methylene-interrupted *cis* double bonds, along with beeswax and *Avicennia officinalis* leaf wax were lipolysed. The results of on-plate lipolysis for 96 min compared well with those obtained by chemical hydrolysis in tubes.

INTRODUCTION

Although porcine pancreatic lipase is known to possess various carboxyl ester-cleaving activities[1,2], it has been only extensively used for the hydrolysis of triglycerides for compositional studies[3–6]. In continuation of our previous studies on enzymatic reactions on thin-layer plates[7–9] the lipolysis of wax esters was undertaken. This on-plate method was thought to be useful in finding the fatty acid and alcohol compositions of milligram amounts of waxes.

EXPERIMENTAL

Synthetic wax

Synthetic wax was prepared by condensation[10] of the acyl chlorides of free

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fatty acids obtained from the hydrolysis of linseed oil and oleoyl alcohol. Linseed oil was hydrolysed by refluxing with 5% potassium hydroxide in methanol for 1 h. The methanol was evaporated, the residue was dissolved in water and non-saponifiables were removed by four extractions with diethyl ether. Free fatty acids were liberated by acidifying with 4 *N* sulphuric acid, extracting with diethyl ether and drying over anhydrous sodium sulphate. The ether was removed and the fatty acids were refluxed with oxalyl chloride in light petroleum. The acyl chlorides were recovered and stored in a desiccator. Oleoyl alcohol (99%, Nu-Chek-Prep., Elysian, MN, U.S.A.) was condensed with the acyl chlorides in the presence of dry pyridine[10]. The wax was recovered, dried and stored. The purity of the synthetic wax was checked by thin-layer chromatography (TLC) and infrared (IR) spectrophotometry and purification was carried out when necessary. Aliquots of the synthetic wax were hydrolysed as described earlier and the free fatty acids were liberated and methylated using diazomethane[11]. Subsequently, the composition of the fatty acid methyl ester of the synthetic wax was determined by gas-liquid chromatography (GLC). Palmitoyl oleate was synthesised from palmitoyl alcohol and oleic acid.

Natural waxes

Wax was extracted from the leaves of *Avicennia officinalis* Linn., grown abundantly in the Sunderban mangrove forest of Eastern India. The fresh leaves were shaken with redistilled hexane for 1 min and filtered. The wax solution was concentrated in a rotary evaporator and purified by preparative TLC on silicic acid layers, using a solvent system of 2% diethyl ether in hexane[12]. The bands were visualised by iodine vapour and those corresponding to authentic wax esters were recovered. Beeswax obtained from bee hives in the Sunderban mangrove forest was purified similarly. The purity of the isolated wax esters was checked by IR spectrophotometry and analytical TLC.

Preparation of lipase solution

The lipase (Sigma type II, crude from hog pancreas; Sigma, St. Louis, MO, U.S.A.) was washed free from lipids by vigorous shaking, twice each with acetone and diethyl ether[6], followed by centrifugation. The washings were discarded and the pellet was dried in a vacuum oven at 30°C for 1 h. A saturated solution of this lipase in 1 *M* Tris buffer (pH 8.2) was prepared and centrifuged and the supernatant was used for the determination of activity. The amount of the soluble protein per ml of this solution was determined by the method of Lowry *et al.*[13]. This amount of the soluble protein was referred to as the amount of lipase. The lipase solution thus prepared in Tris buffer contained 66% (w/v) protein. The activity of this enzyme solution was determined by the method of Luddy *et al.*[6] and found to be 100 units per mg of protein using olive oil as substrate at 37°C. The activity of this lipase solution when palmitoyl oleate was used as substrate was 1 unit per mg of protein. An activity of 1 unit is defined as the amount that will hydrolyse 1 microequivalent of oleic acid from palmitoyl oleate in 1 h at pH 8.2 and 37°C.

Preparation of bile salt and calcium chloride solutions

A 0.1-g amount of dried bile salt (Oxo, London, U.K.) was mixed with 1 ml of water and autoclaved at 15 p.s.i. for 1.5 h. A 10-ml volume of glycerol was added

and the mixture was heated on a water-bath until homogeneous[7]. A 5.0-g amount of calcium chloride dihydrate (E. Merck, Darmstadt, F.R.G.) was dissolved in 100 ml of water.

Lipolysis of wax esters on TLC plates and separation of the products

About 1–4 mg of protein in 0.2 ml of Tris buffer (pH 8.2) was evenly applied as a band on the plate. The band was dried to some extent by a stream of air. Approximately 1–4 mg of wax ester, dissolved in *n*-hexane, was applied evenly on to this enzyme band. The reaction was carried out in incubators at specific temperatures. To avoid excessive loss of water from the reaction zone, the plates, gel-side down, were placed over a large dish of water kept in the incubator. After a stipulated period of incubation, the plate was placed in an ether chamber and developed three times to 2 cm over the line of application to extract the lipids out of the reaction zone. The plate was then developed to 14 cm with *n*-hexane–diethyl ether–acetic acid (90:10:1.5, v/v/v) to resolve the fatty acids, alcohols and unreacted wax ester. The bands were located by iodine vapour, the iodine was evaporated off and the bands were scraped off the plate into small glass columns. The lipids were extracted from the columns with five 2-ml portions of diethyl ether. The solvents were evaporated off under nitrogen and the products were derivatized.

Lipolysis of synthetic wax was also done in vials, following the method of Luddy *et al.*[6]. A 4-mg sample of wax along with 4 mg of lipase, 0.5 ml of bile salt solution, 0.2 ml of calcium chloride solution and 2 ml of Tris buffer (pH 8.2) were put into screw-cap vials, which were shaken at 3000 strokes per min in a wrist shaker for various periods of time. The reactions were stopped by adding an excess of diethyl ether after stipulated periods. The products were extracted by diethyl ether, separated on TLC plates and analysed by GLC.

Methylation of free fatty acids and acetylation of alcohols

The free fatty acids were methylated using an ethereal solution of diazomethane[11]. For acetylation[14] the alcohols were dissolved in dry pyridine, acetic anhydride was added and the mixture was kept in a vial with PTFE-lined screw cap at 100°C for 2 h. The vial was cooled to room temperature and the contents were poured over ice-cold water. The acetates were recovered from this mixture by three extractions with chloroform. The pooled chloroform extract was washed with water, dried over anhydrous sodium sulphate and brought to a small volume by evaporation at low pressure.

Determination of fatty acid and alcohol compositions of waxes by GLC

The fatty acid methyl esters and the alcohol acetates of various waxes were analysed by GLC for the determination of absolute quantities of each of the fatty acids, known weights (*ca.* 50 μg) of methyl pentadecanoate being added to each sample of fatty acid methyl esters. An aliquot of methyl pentadecanoate was reduced by lithium aluminium hydride to pentadecanol and its acetate was prepared as described before. The acetate was purified by preparative TLC and the purity checked by GLC. To each of the alcohol acetate samples was added *ca.* 50 μg of pentadecanol acetate before GLC.

The GLC instrument used was from Pye Unicam (London, U.K.), Model

GCD, with dual column and dual flame-ionization detectors. The columns used were 10% DEGS (diethylene glycol succinate polyester) for fatty acid methyl esters and 3% OV-17 liquid phase for alcohol acetates. Both the liquid phases were coated on Chromosorb W HP, 80–100 mesh, and were packed into 1.8 m × 0.3 cm coiled glass columns. Fatty acid methyl esters were analysed isothermally at 180°C. For acetylated alcohols the temperature was programmed from 220 to 300°C at the rate of 4°C per min. Appropriate authentic standards were used under similar operational conditions. The carrier gas used was nitrogen, with a flow-rate of 60 ml/min for all analyses. Quantifications were done by triangulation and peak-area-normalisation techniques.

RESULTS

Quantitative removal of the free fatty acids and alcohols from the reaction zone

The number of developments with diethyl ether required for the quantitative removal of the lipid reaction products from the reaction zone was established in a manner similar to that reported earlier[7]. It was found that complete removal of the products from the reaction zone was achieved by three extractions.

Effects of bile salt and calcium chloride on the on-plate lipolysis of palmitoyl oleate

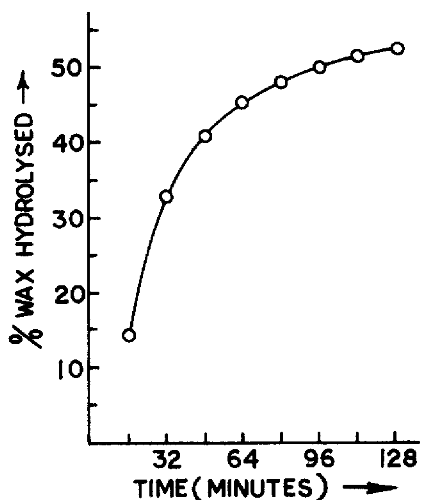
To study the effects of bile salt and calcium chloride, an experiment similar to that described earlier[7] was performed. Four plates were used, 4 mg lipase and 1 mg of palmitoyl oleate being used on each plate. To the first plate was added 0.04 ml of 5% calcium chloride solution and 0.06 ml of bile salt solution. To the second plate was added 0.04 ml of calcium chloride solution, the bile salt solutlion being omitted. The third and the fourth plates contained only the enzyme and the substrate. The enzyme of the fourth plate was made inactive by boiling for 30 min. Incubation was continued for 96 min at 45°C after the addition of substrate and the hydrolysed fatty acids were then estimated by GLC. The results showed that hydrolysis to the extent of 50% was achieved, irrespective of the presence of bile salt and/or calcium chloride.

Time-course study of the lipolysis

The time course of the on-plate lipolysis of palmitoyl oleate is shown in Fig. 1, which indicates that 50% of the substrate was hydrolysed in 96 min. From Fig. 1 it is also evident that in 128 min *ca.* 53% of the palmitoyl oleate has been hydrolysed and the curve becomes almost parallel to the time axis. The same time-course experiment was carried out with the synthetic wax containing various fatty acids esterified with oleoyl alcohol; this produced a similar curve to that shown in Fig. 1. Analysis of the various fatty acids liberated at different time periods indicated that the fatty acid composition at 96 min of enzymatic lipolysis becomes identical to that obtained by chemical hydrolysis of the synthetic wax.

Specificity of lipase to waxes containing unsaturated and saturated fatty acids

The specificity of the lipase to unsaturated and saturated fatty acids was studied using the synthetic wax in which various fatty acids had been esterified with oleoyl alcohol. The saturated fatty acids used were palmitic and stearic acids; the unsaturated fatty acids were oleic, linoleic and linolenic. The wax was lipolysed for various



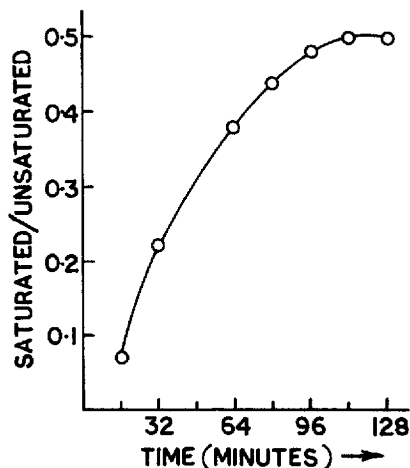


Fig. 1. Time course of on-plate lipolysis of palmitoyl oleate. On each plate 1 mg of lipase and 1 mg of palmitoyl oleate were applied and incubated at 45°C. The amount of wax hydrolysed was estimated by analysing the oleic acid liberated.

Fig. 2. Specificity of lipase on the hydrolysis of synthetic wax containing various saturated and unsaturated acids. On each plate 1 mg lipase and 1 mg wax were applied and incubated at 45°C. The fatty acids liberated at various time intervals were separated, recovered, methylated and analysed by GLC.

periods and the liberated fatty acids were extracted, methylated and analysed by GLC. The ratios of total saturated to unsaturated acids liberated at different times are shown in Fig. 2. The result shows that the ratio gradually increases and becomes steady at 0.48 after 96 min. The composition of free fatty acids and the ratio of saturated to unsaturated fatty acids after 96 min of lipolysis becomes identical to those obtained by chemical hydrolysis (Table I).

TABLE I

FATTY ACID COMPOSITIONS (% w/w) OF BEESWAX, LEAF WAX AND SYNTHETIC WAX BY LIPASE AND CHEMICAL HYDROLYSIS

Analyses were performed by GLC using a 10% DEGS column; nitrogen flow-rate, 60 ml/min; oven temperature, 180°C.

*Component acids**	*Chemical hydrolysis*			*Lipase hydrolysis*		
	Beeswax	*Leaf wax*	*Synthetic wax*	*Beeswax*	*Leaf wax*	*Synthetic wax*
12:0	–	0.8	–	–	5.4	–
14:0	2.0	1.0	–	2.5	1.4	–
16:0	78.6	37.0	23.2	77.5	37.5	23.5
18:0	14.8	4.0	8.8	13.8	4.8	9.5
18:1	2.7	39.0	25.0	3.4	38.3	26.0
18:2	0.7	16.0	11.0	1.2	15.8	10.0
18:3	–	–	32.0	–	–	31.0
20:0	1.2	2.2	–	1.6	1.8	–

* First figure represents the carbon chain length and the second, the number of double bonds.

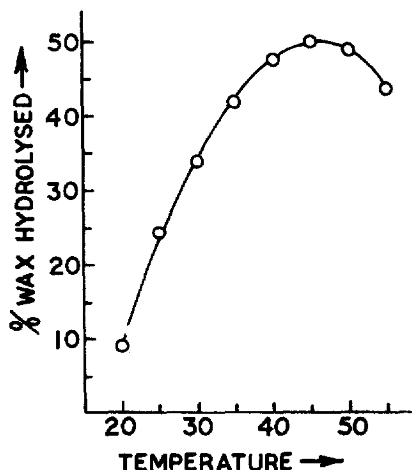


Fig. 3. Lipolysis of palmitoyl oleate at various temperatures. On each plate 1 mg lipase and 1 mg substrate were taken and the plates were incubated at various specified temperatures. Each plate was incubated for 96 min. The oleic acid liberated at various temperatures was estimated by GLC and the extent of the hydrolyses were calculated.

Effect of incubation temperature on lipolysis

The extent of lipolysis using palmitoyl oleate was studied at various temperatures between 20 and 55°C. The results (Fig. 3) show that, for 96-min incubations at various temperatures, the extent of hydrolysis increases gradually and reaches *ca.* 50% at 45°C, decreasing above this temperature.

Application of lipolysis to some natural waxes on TLC plate

The method of lypolysis was applied to beeswax and *A. officinalis* leaf wax on TLC plates. After lipolysis for 96 min at 45°C, the fatty acids and alcohols were recovered as described earlier, derivatized and analysed by GLC and the compositions were determined. Chemical hydrolyses of the same waxes were also carried out and the compositions of the fatty acids and alcohols were determined. The fatty acid and alcohol compositions of the waxes are shown in Tables I and II, respectively.

DISCUSSION

It has been pointed out that porcine pancreatic lipase has various carboxyl esterase activities[1,2]. Although pancreatic lipase basically hydrolyses the triacyl glycerol ester bonds, it is also known to hydrolyse the acyl ester linkages of phospholipids[15,16], cholesterol esters[17] and galactolipids[18]. The first observation, made by Balls and Matlack[3], was that pancreatic extract digested all esters of primary alcohols, whereas secondary and tertiary esters were hardly attacked. Brockerhoff[20] compared the speed of hydrolysis of various esters by pancreatic lipase. Short-chain halogenated *n*-alcohols and various aromatic alcohols with and without polar substitutions were esterified with oleic acid to obtain various wax esters. It was established conclusively by Brockerhoff[20] that, the higher the inductive effect, the higher was the rate of hydrolysis.

The present study was carried out to make use of the carboxyl esterase activity of pancreatic lipase in the analysis of natural waxes. For this purpose, in continuation of our study of lipolysis on TLC plates[7–9], we elaborated this technique for compo-

TABLE II

ALCOHOL COMPOSITIONS (% w/w) OF BEESWAX AND LEAF WAX BY LIPASE AND CHEMICAL HYDROLYSIS

Alcohol acetates were analysed by GLC using a 3% OV-17 column; nitrogen flow-rate, 60 ml/min; oven temperature, 220°C at the start, programmed at the rate of 4°C/min up to 300°C.

*Component alcohols**	*Chemical hydrolysis*		*Lipase hydrolysis*	
	Beeswax	*Leaf wax*	*Beeswax*	*Leaf wax*
14:U**	–	0.6	–	0.5
14:0	0.9	0.6	0.7	1.0
16:0	0.9	1.0	1.0	0.8
17:U	–	1.7	–	1.0
17:0	0.5	1.8	0.2	2.0
18:U	1.0	–	0.8	–
18:0	3.0	2.2	3.0	2.5
20:U	1.0	1.6	1.2	1.2
20:0	2.6	3.1	3.0	3.0
21:U	–	0.8	–	0.5
21:0	0.9	4.9	1.1	5.0
22:U	–	0.3	–	0.5
22:0	1.0	2.5	1.3	2.8
23:U	–	0.3	–	0.5
23:0	–	2.2	–	2.0
24:0	48.5	48.0	47.7	47.0
25:0	–	0.8	–	1.0
26:U	–	0.5	–	0.4
26:0	13.7	8.3	14.5	8.0
27:0	–	0.8	–	0.5
28:U	1.3	1.0	1.0	1.1
28:0	4.2	6.3	5.0	5.8
29:0	–	0.5	–	0.8
30:0	5.8	3.8	5.6	3.9
31:0	–	0.1	–	0.8
32:0	8.8	4.0	8.5	3.9
34:0	5.2	2.3	4.8	3.5
36:0	0.7	–	0.6	–

* First figure represents the carbon chain length and the second the unsaturation.
** U represents unsaturated alcohol.

sitional studies of natural waxes. The technique has advantages[7–9] over conventional chemical hydrolysis or conventional lipolysis of synthetic wax in vials.

The time-course study of lipolysis (Fig. 1) shows that 50% hydrolysis occurs at 96 min, when the composition of the fatty acids from various waxes becomes identical to those by chemical hydrolysis (Table I). The wax esterase activity in the pancreatic lipase is very low compared to that of triglycerides[7]. A time-course study in vials[6] showed similar results with synthetic wax.

From the increase with time in the ratio of saturated to unsaturated acid (Fig. 2), it can be concluded that the pancreatic lipase specifically hydrolyses during the

first phase of the reaction the waxes with unsaturated acids at a faster rate than those containing saturated acids. In the later phase of lipolysis, the value of this ratio becomes steady and finally at 96 min reaches *ca.* 0.5. The finding that the same value of this ratio is obtained by chemical hydrolysis indicates that the representative fatty acid composition of the wax is only obtained after lipolysis for 96 min.

The optimum temperature for the lipolysis of wax esters is 45°C, as is indicated in Fig. 3.

The optimum value of the enzyme-to-substrate ratio for the lipolysis of wax ester, determined previously[7] as 1:1, is bound to be quite suitable in this study.

CONCLUSIONS

The method of lipolysis of wax esters was used to study both the fatty acid and alcohol compositions of two natural waxes, *viz.* beeswax and *A. officinalis* leaf wax, both collected from Sunderban mangrove forest. The chemical hydrolyses of these two waxes were also reformed simultaneously. A comparison of the fatty acid and alcohol compositions of these two waxes obtained by chemical and enzymatic hydrolysis has been made. It is found that there is good agreement of results by the two methods and that the lipolysis of natural wax esters on TLC plates can be used for compositional studies.

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