ENZYMATIC REACTIONS ON THIN-LAYER CHROMATOGRAPHIC PLATES

II. PHOSPHOLIPASE A$_2$ HYDROLYSIS OF PHOSPHATIDYLCHOLINE AND SEPARATION OF THE PRODUCTS ON A SINGLE PLATE

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SUMMARY

   A procedure for the phospholipase A$_2$ hydrolysis of phosphatidylcholine on a thin-layer chromatographic plate and subsequent separation of the products on the same plate is described. A 0.2–0.8-mg amount of Russell's viper venom (phospholipase A$_2$) in 0.2 ml of 0.005 M calcium chloride solution was applied on a 0.5-mm silica gel G plate as a band over which 2–5 mg of egg phosphatidylcholine in 0.2 ml of diethyl ether containing 5% of methanol was evenly applied. After the reaction had proceeded for 15–20 min in a diethyl ether-saturated chamber at 25°, the plate was developed with chloroform–methanol–water (65:25:4). The bands were identified and their contents extracted. The extent of hydrolysis under different reaction conditions was evaluated from the amount of lysophosphatidylcholine formed. Approximately 74.6% (maximum) conversion was obtained within 15 min at 25° using a substrate to enzyme ratio of 4:1. The acyl group distributions in the 1- and 2-positions of hen egg phosphatidylcholine obtained from the gas–liquid chromatographic analysis of the methyl ester corresponding to the lyso and free fatty acid band agreed with those obtained by the method of Wells and Hanahan. The method is also applicable to phosphatidylethanolamine.

INTRODUCTION

   Studies over the last decade have revealed that phospholipids of membranes from different animal tissues consist of molecular species differing in the fatty acid distributions at the 1- and 2-positions of the diacylglycerophosphatide molecules[1]. It has been suggested that the specific distributions of the fatty acid components at these two positions not only reflect the biosynthetic capabilities of the tissue membrane

but also are the products of a regulatory mechanism for maintaining the appropriate lipid–lipid or lipid–protein interactions as required for optimal functioning of the particular membrane concerned[2]. Thus an understanding of the nature and distributions of different fatty acyl groups in phospholipids is assuming increasing importance. Hydrolysis of the acyl group attached at a specific position by stereospecific phospholipase, viz., phospholipase A_2[3-6], separation of the products and evaluation of the compositions of fatty acids in the lysophospholipid and free fatty acid fractions is the basis of finding the distributions of acyl groups in the 1- and 2-positions of phospholipids[7-10].

     Recently we developed a method[11] in which hydrolysis of the acyl group attached to the 1- and 3-positions of triglycerides by pancreatic lipase and separation of the reaction products were carried out on a single thin-layer chromatographic (TLC) plate. This paper deals with the development of a similar method in which phospholipids are hydrolysed at the 2-position by phospholipase A_2 (Russell's viper venom) and the reaction products are separated on the same TLC plate. The products of hydrolysis can easily be isolated and their fatty acid compositions can be evaluated by gas–liquid chromatography (GLC).

     The extent of hydrolysis in relation to different reaction conditions has been measured. The method has been used to establish the distributions of the acyl groups at 1- and 2-positions of phosphatidylcholine from hen egg. An extent of hydrolysis comparable to that of phosphatidylcholine under optimal conditions has also been observed with phosphatidylethanolamine.

EXPERIMENTAL

Solvents

     All solvents were of analytical-reagent grade, and were dried and redistilled. Diethyl ether was freed from peroxide before being dried and distilled.

Reference lipids

     Phosphatidylcholine (PC) (egg), lysophosphatidylcholine (LPC) (egg) and phosphatidylethanolamine (bovine), all of 99% purity, were purchased from Applied Science Labs. (State College, Pa., U.S.A.).

Reagents

     Source of phospholipase A_2 (E.C. 3.1.1.4). Lyophilized venom of Russell's viper (Vipera russelli) was purchased from the CSIR Centra for Biochemicals (University of Delhi, New Delhi, India).

     Preparation of venom solution, determination of its protein content and assay of the activity of phospholipase A_2 in the solution. The lyophilized venom was shaken with the appropriate volume of 0.005 M calcium chloride solution ($CaCl_2 \cdot 2H_2O$, G.R. grade; E. Merck, Darmstadt, G.F.R.) (2 mg of venom per millilitre). The venom dissolved completely in the calcium chloride solution. This solution was used for all experiments. The protein content per millilitre of the solution was determined according to Lowry et al.[12] and the activity of phospholipase A_2 was assayed according to the method of Magee and Thompson[13] as modified by Dawson[14]. Venom was found to be composed solely of soluble protein. The activity of phospholipase A_2 was found

to be 1.2 units per milligram of protein (1 unit will hydrolyse 1 μmole of phosphatidylcholine to lysophosphatidylcholine per minute at pH 7.2 and 30°).

Plate preparation

Glass plates (20 × 14 cm) were coated with 0.5-mm layers of silica gel G (E. Merck), activated at 110° for 1 h and stored in a desiccator. Before use the plates were pre-developed in diethyl ether to remove any organic contaminants to the top of the plate, from where they were removed by scraping off a narrow band of the adsorbent.

Phospholipase A_2 hydrolysis of egg phosphatidylcholine and resolution of the products on the same TLC plate

The required volume of venom solution (0.2–0.4 ml) containing 0.4–0.8 mg of venom protein was applied as a band on the preparative TLC plate 2 cm from one of the shorter edges. The plate was held horizontally in the air draught from an electric fan for 10 min to remove most of the water from the band. The required amount of egg PC (2.5–5 mg) in peroxide-free diethyl ether containing 5 % of methanol was applied as evenly as possible over the enzyme band. The plate was immediately placed in a TLC chamber saturated with diethyl ether vapour (a beaker containing the solvent was placed in the chamber for this purpose) and kept at a specific temperature in order to prevent the evaporation of ether from the application zone. After a stipulated time (15–20 min) the plate was transferred quickly into another saturated TLC chamber and developed to 14 cm from the line of application with chloroform–methanol–water (65:25:4). The different bands on the developed chromatogram were located with iodine vapour (Fig. 1), identified by comparing their R_F values with those
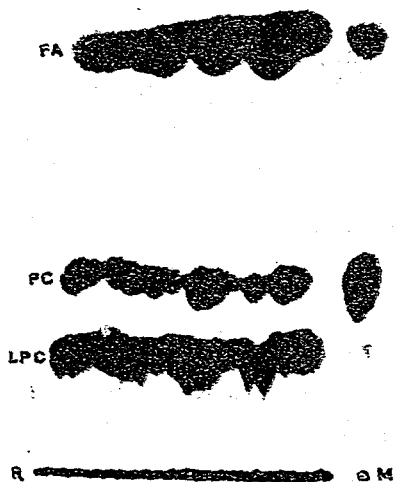

Fig. 1. Hydrolysis of egg PC by phospholipase A_2 and resolution of the reaction products on the same TLC plate. TLC plate: 14 × 20 cm, 0.5-mm silica gel layer. Reaction conditions: 0.4 mg of venom (Russell's viper) equivalent to 0.48 unit (see text) of phospholipase A_2 applied as band over which 4.8 mg of egg PC were evenly applied; reaction temperature, 25°; reaction time, 15 min; developing solvent, chloroform–methanol–water (65:25:4); detection, treatment with iodine vapour followed by spraying with 0.5 % starch solution. R = Reaction zone; M = mixture of reference LPC, PC and linoleic acid.

TABLE I

RECOVERY OF LPC FROM THE REACTION ZONE

Set of experiments	Materials applied in sequence on the chromatographic plate		LPC recovered (μmole)		LPC recovered $(R - L)$ (μmole)	Recovery of LPC (%)
	Left-hand half	Right-hand half	From left-hand half (L)	From right-hand half (R)		
1st	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 4.60 μmole of LPC	1.53 ± 0.02	6.09 ± 0.10	4.56 ± 0.10	99.13 ± 2.17
2nd	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 9.30 μmole of LPC	1.52 ± 0.03	10.79 ± 0.24	9.27 ± 0.24	99.68 ± 2.58
3rd	0.40 mg of lipase, 2.06 μmole of egg PC	0.40 mg of lipase, 2.06 μmole of egg PC, 13.90 μmole of LPC	1.53 ± 0.04	15.22 ± 0.07	13.69 ± 0.08	98.49 ± 0.58

of known standards and marked. The LPC band was then scraped off the plate, transferred to a mini-column and the lipids were recovered from the adsorbent by extraction with three 3-ml portions of chloroform–methanol–water (1:1:0.2). The solvents were evaporated under reduced pressure, removing the last traces by flushing with nitrogen. The lipids were finally dissolved in a known volume of the same solvent mixture for the determination of LPC. Where determinations of fatty acid (FA) composition were required, the FA bands were extracted in a similar manner with diethyl ether. The solvent was removed under nitrogen and the lipids were redissolved in a known volume of chloroform and kept under nitrogen in the cold for further analysis.

Determination of LPC

LPC was estimated spectrophotometrically as micromoles of phosphorus according to Ames[15]. From this result the molar percentage hydrolysis of PC was calculated.

Isolation of PC from hen egg

Yolks from fresh hen eggs were collected, homogenized with half their volume of distilled water and the lipids extracted by the procedure of Bligh and Dyer[16]. Phospholipids were isolated by silicic acid (100 mesh; Mallinckrodt, St. Louis, Mo., U.S.A.) column chromatography[17]. Pure PC was isolated from hen egg phospholipids by preparative TLC using chloroform–methanol–water (65:25:4) as the developing solvent. Bands were detected with iodine vapour, the R_F values were compared with those of known standards and the lipids were recovered from the adsorbent by elution with chloroform–methanol–water (1:1:0.2). The isolated material produced a single spot of phospholipid with an R_F value identical with that of reference PC spotted on the same TLC plate.

Identification of FA components by GLC

The FA composition of the phospholipids and the products of their on-plate lipolysis by phospholipase A_2 were determined by the GLC analysis of the corresponding mixed methyl esters prepared by the methanol–sulphuric acid method[18,19]. A dual-column F & M Model 700R analytical gas chromatograph with a flame-ionization detector (FID) was used. Chromatograms were obtained on 6 ft. × 1/8 in. stainless-steel columns packed with 10% EGSS-X coated on 100–120-mesh Gas-Chrom W (Applied Science Labs.) at 160°. The carrier gas (nitrogen) flow-rate was kept at 40 ml/min. Components were identified by comparing their retention times with those of authentic standards. Compositions were calculated from peak areas obtained by the triangulation method and corrected by multiplication by the appropriate calibration factors.

RESULTS

The results in Table I show the recovery of LPC from the reaction zone. Twelve chromatographic plates divided into three equal sets were used in these experiments. Each plate was divided into two halves and on each half 0.4 mg venom was applied as bands. On the left-hand half of all the plates 2.06 μmole of egg PC were applied over the enzyme bands, but on the right-hand half of the plates of the first, second

and third sets, in addition to 2.06 μmole of egg PC, 4.6, 9.3 and 13.9 μmole of LPC were added over the enzyme bands. Reactions were carried out at 25° for 15 min. The contents of all the LPC bands were measured. The amounts of LPC obtained from the left-hand halves were due to the hydrolysis of 2.06 μmole of PC and those from the right-hand halves from the hydrolysis of 2.06 μmole of PC and the added LPC. The difference between these two results was obviously the amount of LPC recovered. The results show that up to at least 13.9 μmole (ca. 7.22 mg) of LPC added, the removal from the reaction zone was almost quantitative. As LPC was the most polar of all the lipid components on the chromatogram, it can be assumed that other products were also removed quantitatively from the reaction zone.

The results of the time course of on-plate lipolysis (Fig. 2) show that at a substrate to enzyme ratio of 12:1, between 2.5 and 15.5 min the extent of hydrolysis increased from 17.0 $\pm$ 1.0 to 56.1 $\pm$ 0.5% and remained steady at about this level (56.3 $\pm$ 0.5%) up to 30.5 min. The conversion can be increased by decreasing the substrate to enzyme ratio, as is clear from Fig. 3. The maximal conversion of approximately 74.6% can be achieved with the present enzyme sample when this ratio is 4 or less. When the method is to be used for either the preparation of pure lyso compound or the determination of acyl group distributions in phospholipids, a lower ratio is obviously to be preferred, but care should be taken not to overload the TLC plate.

The extents of lipolysis within 15 min using 0.4 mg of venom and 6.19 μmole of egg PC at different reaction temperatures between 5 and 35° were measured. The results (Fig. 4) show that the extent of hydrolysis increased rapidly up to 20°, at which temperature ca. 53.0 $\pm$ 0.2% of egg PC was hydrolysed within 15 min. On a further increase in temperature up to 30°, this value increased very slowly to 56.1 $\pm$ 0.5%, probably owing to an increased rate of evaporation of diethyl ether from the reaction
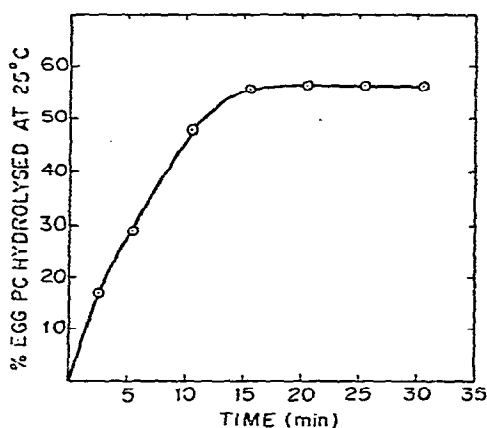

Fig. 2. Time course of on-plate lipolysis of egg PC. Venom from Russell's viper (0.4 mg) was applied as a band over which 4.8 mg of egg PC (ca. 6.19 mole) were added throughout the band as quickly as possible. Reactions at 25° were carried out for 2.5, 5.5, 10.5, 15.5, 20.5, 25.5 and 30.5 min, the time being calculated from just after the complete addition of PC on the enzyme band to the complete coverage of the reaction zone by the developing solvent. The amounts of PC hydrolysed were evaluated from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within $\pm$0.8.
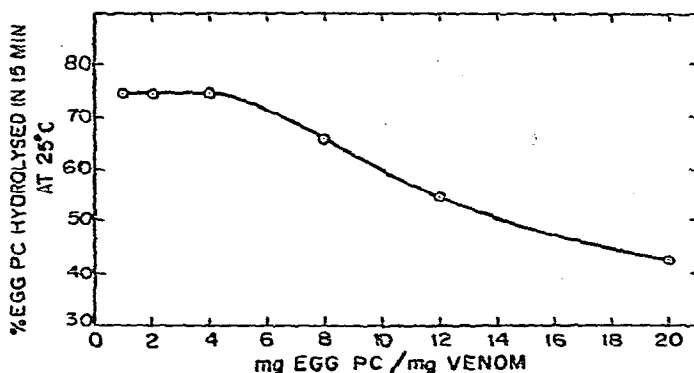
Fig. 3. Effect of substrate concentration per milligram of venom on the extent of lipolysis. On each of six sets (four in a set) of TLC plates 1 mg of venom was applied. The amounts of egg PC applied were 1, 2, 4, 8, 12 and 20 mg. The reaction was allowed to proceed for 15 min at 25° and the amounts of PC hydrolysed were measured from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within ± 0.9.
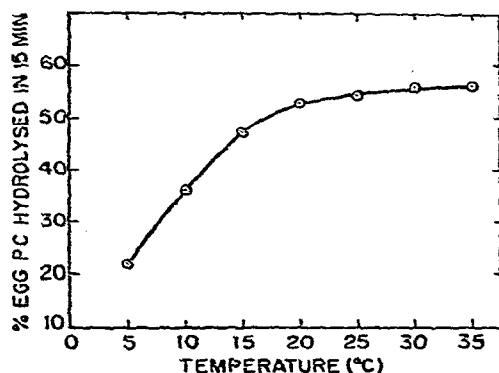

Fig. 4. Effect of temperature on the extent of hydrolysis. Venom (0.4 mg) and egg PC (4.8 mg) (ca. 6.19 mole) were applied, in sequence, as a band on each of seven sets of TLC plates. The reaction was carried out for 15 min at 5°, 10°, 15°, 20°, 25°, 30° and 35° from the first to the seventh set. The amounts of PC hydrolysed were calculated from those determined for LPC. Each point on the curve is the mean of the results of four experiments; the deviation from the mean for all the points was within ±0.6.

zone. It was shown by Hanahan[20] that during this enzymatic reaction an ether-soluble enzyme–substrate complex is formed.

The positional distributions of fatty acids in hen egg PC were determined. Hen egg PC was subjected to on-plate enzymatic hydrolysis (1 mg of venom per 5 mg of substrate) and the reaction products were separated and isolated. The fatty acid compositions of LPC and free FA were determined by GLC and the positional compositions are given in Table II. The same compositions were also determined by the GLC analysis of the products obtained by the lipolysis of hen egg PC according to the method of Wells and Hanahan[6], and are reported in Table II for comparison. The results show that the compositional data obtained by the two methods were in close agreement. The major molecular species of hen egg PC can be calculated from the compositions in Table II assuming a 1-random–2-random distribution of acyl groups.

TABLE II

POSITIONAL DISTRIBUTION OF FATTY ACIDS IN HEN EGG PC

Position	Method of calculation	Fatty acid composition							
		14:0	16:0	16:1	18:0	18:1	18:2 (n—6)	20:4 (n—6)	22:6
PC*	—	1.2	31.5	2.4	16.6	34.4	10.5	3.4	tr**
1	This work	1.7	58.7	tr**	31.7	6.1	1.2	0.6	—
	Ref. 6	1.5	58.3	tr**	32.5	5.8	1.0	0.9	—
2	This work	0.1	1.7	4.1	0.6	65.2	21.4	6.9	tr**
	Ref. 6	0.1	1.2	3.3	tr**	66.4	22.8	6.2	tr**

* Initial phosphatidylcholine.
** tr = less than 0.1%.

It was found that phosphatidylethanolamine can be effectively subjected to on-plate hydrolysis by snake venom phospholipase A_2. Experiments with 2.4 mg of phosphatidylethanolamine and 0.2 mg of venom at 25° showed that $56.5 \pm 1.1\%$ of the lipids can be hydrolysed within 15 min.

DISCUSSION

A number of methods have been established for enzymatic deacylation at the 2-position in phospholipids[3-6]. Although good results for the positional distribution of acyl groups can be obtained by these methods, they involve a number of steps. For example, those of Haverkate and Van Deenen[5] and Wells and Hanahan[6] require vigorous shaking of the reaction mixture, evaporation of the solvents under nitrogen, separation of the products by TLC and evaluation of acyl group distributions by GLC. The time required is several hours.

In the present method a number of steps have been avoided by performing the enzymatic reaction and the separation of the products on the same TLC plate. Thus the time required is considerably reduced and the losses due to transfers are diminished.

The close agreement between the positional distributions of acyl groups in hen egg PC obtained by the present on-plate method and those obtained by Wells and Hanahan[6] indicates that the on-plate method is adequate for the determination of above distribution values. This method can also be used for milligram-scale preparations of pure lyso compounds.

ACKNOWLEDGEMENTS

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