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Note

Assay of phospholipase D on a thin-layer chromatographic plate

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Lipolytic enzymes play an indispensible role in the biological turnover of lipids. The unique property of this class of enzymes is their ability to act at interfaces on substrates (lipids) that are insoluble in water. Therefore, standard biochemical techniques generally used for assaying soluble enzymes would not be suitable in this instance. In connection with our work on lipolytic enzymes, we have felt a need to develop a simple and rapid assay for analysing this class of enzymes.

Thin-layer chromatography (TLC) can be used to study the actions of lipolytic enzymes, which convert small organic solvent-soluble substrates into other compounds of similar nature and solubility. We selected for our initial study phospholipase D, which catalyses the hydrolysis of the phosphate diester bond of phospholipids and yields phosphatidic acid and the head group[1–5]. The former is soluble in organic solvents and the latter in water, and are detectable by thin-layer and paper chromatography, respectively. In this paper we describe a rapid TLC technique for assaying phospholipase D. The dependence of the extent of hydrolysis on reaction parameters such as the amount of enzyme and substrate, time and temperature has been evaluated. A similar type of study with crude pancreatic lipase on triglyceride has been reported recently[6].

EXPERIMENTAL

Materials

Distilled chloroform (analytical-reagent grade), methanol (analytical-reagent grade), calcium chloride (1 *M* solution), sodium acetate buffer (1 *M* solution, pH 5.6), egg phosphatidylcholine, phosphatidylethanolamine and phospholipase D from spinach were used.

Isolation of phospholipase D from spinach

Phospholipase D was isolated from spinach leaves according to the procedure of Kates[7]. It was then lyophilized and the dry, powdered mass was stored at −20°.

Isolation of egg phosphatidylcholine (PC) and phosphatidylethanolamine (PE)

Isolation of egg phospholipid was carried out according to the procedure of Wells and Hanahan[8] and then PC and PE were separated by column chromatography.

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Preparation of TLC plate

Each plate (14 × 20 cm) was coated with a 0.35-mm layer of silica gel G (Centron, Bombay, India) in the usual manner and activated at 110° for 1 h. After activation it was stored in a desiccator containing anhydrous calcium chloride.

Lipolysis of phosphatidylcholine on TLC plate and isolation of phosphatidic acid

A 1-mg amount of PC was mixed with 0.02 ml of 1 *M* calcium chloride solution and 0.02 ml of 1 *M* sodium acetate buffer (pH 5.6). The mixture was sonicated for 3 min and spotted as a band on a preparative TLC plate. An amount of protein (1–3 mg) in water (0.05–0.1 ml) was added over the band. The plate was kept at 37° for several hours and the band was kept moist with water in order to prevent drying or cementing. After the required time the reaction was stopped by adding 0.1 ml of 0.5 *M* hydrochloric acid. The plate was air-dried and developed using the solvent system chloroform–methanol–water (65:25:4). The reaction product (phosphatidic acid) and the unreacted starting material (PC) were detected with iodine vapour and molybdenum blue. The bands were identified by comparison with standard samples that had been spotted on one side of the same TLC plate. After identification they were scraped off, extracted three times with chloroform–methanol–water (65:25:4), dried under vacuum, redissolved in known volume of chloroform and then determined colorimetrically[9].

Optimum time required for phospholipase D reaction on TLC plate

To determine the optimum time for the assay of phospholipase D on TLC plates we used six preparative TLC plates. For each plate the protocol used was substrate, PC (1 mg), 1 *M* calcium chloride solution (0.02 ml), 1 *M* sodium acetate buffer (0.02 ml, pH 5.6) and protein (3 mg) in 0.1 ml of water. The reaction was carried out for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 h on the six plates. After the appropriate time each reaction was stopped by adding 0.5 *M* hydrochloric acid. Each plate was developed with the same solvent system and the bands were identified, extracted and estimated colorimetrically[9] as before.

Optimum protein concentration for phospholipase D reaction on TLC plate

To find the optimum concentration for the lipase reaction we used the same protocol as above but with different amounts of enzyme, *i.e.*, 1.0, 1.5, 2.0, 2.5 and 3.0 mg, on five TLC plates. Each reaction was carried out for 3 h and stopped by acidification as above. The product and the unreacted material were separated and determined colorimetrically[9] as before.

Assay of phospholipase D on TLC plate using PE as substrate

A similar reaction was carried out with PE liposome (1 mg of PE in the assay buffer as above) and protein (3 mg) for 3 h at 37°. The products were separated by TLC and determined colorimetrically[9].

Assay of phospholipase D in presence of diethyl ether

To a solution of PC (1.5 mg) in diethyl ether (0.5 ml), 1 *M* sodium acetate buffer (pH 5.6) (0.04 ml), 1 *M* calcium chloride solution (0.04 ml), enzyme (1.5 mg in 0.6 ml

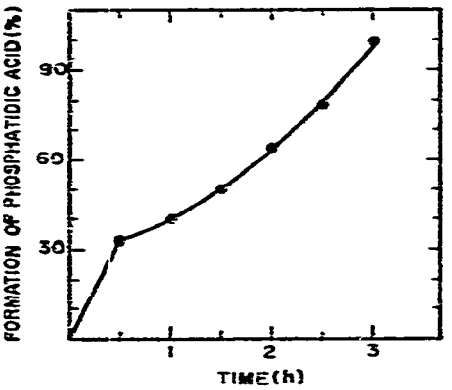

Fig. 1. Formation of phosphatidic acid at different time intervals.

of distilled water) and water (0.32 ml) were added and the mixture was incubated at 37°. TLC analysis showed that the reaction was complete after 3 h.

RESULTS

Fig. 1 and Table I show that the reaction is completed in 3 h at 37°. The reaction is very rapid up to 30 min (when the yield of phosphatidic acid is 33.3%) and then decreases. Similarly, Fig. 2 and Table II show that the reaction is completed when amount of protein is 2.5 mg for 1 mg of PC.

Thus ,the reaction of phospholipase D with PC on the TLC plate is completed in 3 h at 37° when the ratio of the amount of substrate to that of protein is 1:2.5 (w/w). Under similar conditions, PE is completely converted into phosphatidic acid in 3 h at 37° when the substrate to protein ratio is 1:3.

TABLE I

PERCENTAGE OF PHOSPHATIDIC ACID FORMED ON TLC PLATE AT DIFFERENT TIME INTERVALS

Results are means of five experiments.

Plate No.	*Time (h)*	*Phosphatidic acid (%)**
1	0.5	33.3
2	1.0	40.0
3	1.5	50.0
4	2.0	64.3
5	2.5	78.6
6	3.0	~100.0

* Standard ± deviation: 5%.

DISCUSSION

Phospholipids are hydrophobic and the enzyme phospholipase D is water soluble. It is difficult to carry out such heterogeneous reaction on a TLC plate. To overcome this difficulty, the PC or PE suspension is sonicated in the assay buffer

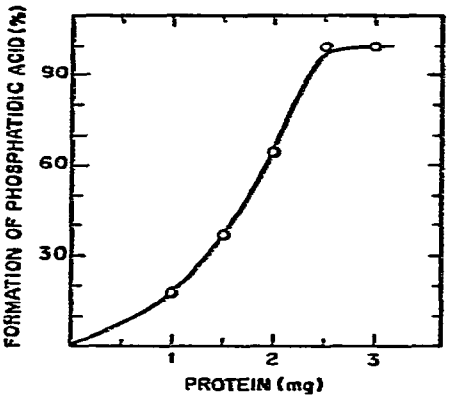

Fig. 2. Formation of phosphatidic acid with different amounts of protein.

TABLE II

PERCENTAGE OF PHOSPHATIDIC ACID FORMED ON TLC PLATE WITH DIFFERENT AMOUNTS OF PROTEIN

Results are means of five experiments.

Plate No.	*Amount of enzyme (mg)*	*Phosphatidic acid (%)**
1	1.0	18.0
2	1.5	36.5
3	2.0	64.7
4	2.5	~100.0
5	3.0	100.0

* Standard deviation: ± 5%.

used for the phospholipase D reaction. The vesicles (liposomes) thus formed are water soluble and are spotted on the TLC plate. This enzymatic reaction usually takes place in the interfacial surface area produced by a solubilizer and diethyl ether[10] is generally used for this purpose. However, in our work there was no need to use a solubilizer because the liposome of PC or PE exposes an extensive surface area for the enzyme reaction.

All of the steps from the enzymatic reaction to the separation of the reaction products are carried out on a single preparative TLC plate, and there is less chance of decomposition of phosphatidic acid as the reaction and the product separation are effected on the same TLC plate within a short time. An advantage is that phospholipase D can be easily detected in the cell homogenate of any prokayote, eukaryote or plant.

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