Journal of Chromatography, 210 (1981) 544–549
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,731

Note

Enzymatic reactions on thin-layer chromatographic plates

III. Phospholipase C hydrolysis of phosphatidylcholine and separation of the products on a single plate; an assay method for the enzyme

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(First received November 17th, 1980; revised manuscript received February 10th, 1981)

The current procedures for determining the distribution of molecular species in a particular phospholipid include steps involving stereospecific hydrolysis by lipase and phospholipases[1–4] and chromatographic separation and isolation of the products. A problem in these enzymatic hydrolyses is the insolubility of the substrate in water. Chemical solubilizers such as bile acids[5], vigorous shaking or preferably sonication[6,7] of the reaction mixture are used to bring about the intimate contact between the substrate and the enzyme which is required for sastisfactory progress of these two-phase reactions. Development by Dutta and co-workers[8,9] of quick and efficient methods for stereospecific hydrolysis of triglycerides and glycerophosphatides by pancreatic lipase and phospholipase A_2 on thin-layer chromatographic (TLC) plates, and separation and isolation of the products through development of such plates, has largely removed the difficulty mentioned. Mandal *et al.*[10] used this technique to develop an assay method for phospholipase D.

We now present an on-plate method for hydrolysis of phosphatidylcholine by phospholipase C, separation and isolation of the reaction products and an assay method for the enzyme.

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 and enzyme

Phosphatidylcholine (PC) (99% pure) (egg) and *sn*-1,2-diolein were purchased from Applied Science Labs. (State College, PA, U.S.A.). Phospholipase C (E.C. 3.1.4.3) lyophilized powder from *Clostridium perfringens* (*Cl. welchii*), Type 1, was purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of enzyme solution and determination of its protein content

The lyophilized powder (2 mg/ml) was shaken with the appropriate volume of 0.1 *M* Tris buffer (pH 7.2) containing 0.02 *M* $CaCl_2$ ($CaCl_2 \cdot 2H_2O$, G.R. grade; E.

0021-9673/81/0000–0000/$02.50

Merck, Darmstadt, G.F.R.). The protein content of this solution, used for all the experiments, was found to be 1.76 mg/ml[11].

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

A 0.2–0.4 ml volume of the enzyme solution was applied as a band on a glass plate (14 × 20 cm) coated with 0.5 mm of silica gel G (E. Merck) which was activated at 110°C for 1 h before use. The plate was held horizontally near an electric fan for a few minutes to remove water from the band. The required amount of egg PC (1–2 μmole), in an appropriate volume of a mixture of peroxide-free diethyl ether and distilled methanol (98:2)[12], was applied evenly over the enzyme band. The plate was immediately placed in a TLC chamber saturated with diethyl ether vapour (a beaker

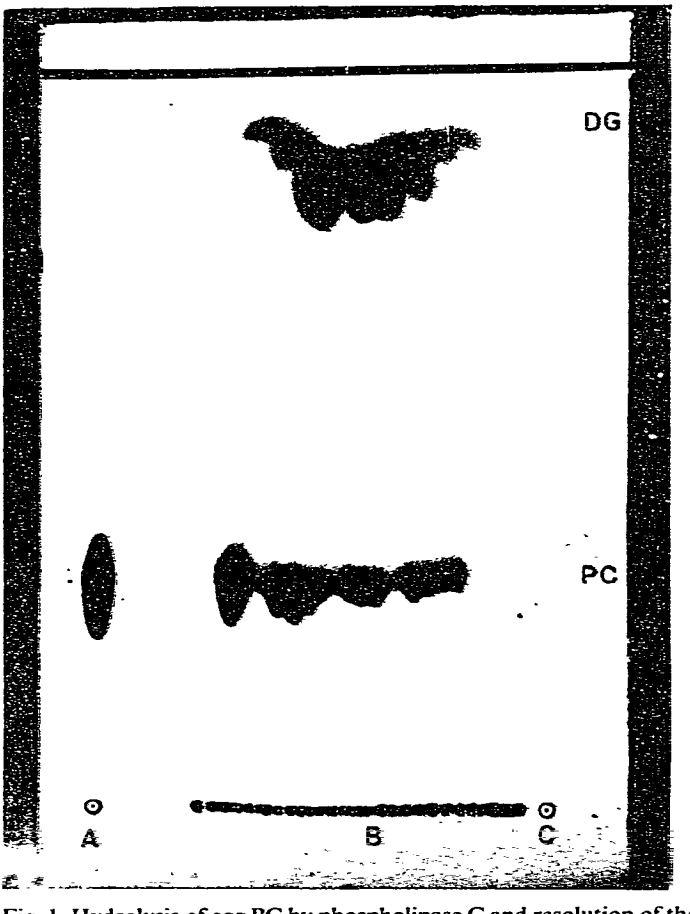

Fig. 1. Hydrolysis of egg PC by phospholipase C and resolution of the reaction products on the same TLC plate. TLC plate: 20 × 14 cm, 0.5 mm of silica gel. Reaction conditions: 2 mg of phospholipase C applied as a band over which 4.0 μmole of egg PC were evenly applied; temperature, 25°C; time, 1 h. Chromatographic development and detection: developing solvent, chloroform–methanol–water (65:25:1); detection, treatment with iodine vapour followed by spraying with 0.5% starch solution. A = Reference egg PC; B = reaction zone containing the phosphorylcholine; C = reference *sn*-1,2-diolein; DG = *sn*-1,2-diglycerides.

of this solvent was placed in the chamber) and kept at 25°C. After some time (1 h) the reaction can be stopped by exposing the plate to HCl vapour for 1 min in a closed TLC chamber. The acid from the plate was removed with an air drought from an electric fan. The plate was then developed in a saturated chamber, up to 14 cm from the line of application with the solvent mixture chloroform–methanol–water (65:25:1). The *sn*-1,2-diglycerides produced and the unreacted PC on the chromatogram were located with iodine vapour, identified by comparing their R_F values with those of known standards and marked. The phosphorylcholine part of the products remained at the reaction zone (Fig. 1). For preparative purposes, stopping the reaction with HCl should be avoided in order to prevent acyl migration.

Estimation of PC hydrolysis

The amount of PC hydrolysed was estimated from either the unreacted PC or the phosphorylcholine formed.

For the estimation from unreacted PC, the corresponding band was scraped off and the lipids were recovered by extraction with three 3-ml portions of chloroform–methanol (1:1) containing 10% water, according to Webb and Mettrick[13]. The solvents were evaporated under reduced pressure and the lipids re-dissolved in a known volume of chloroform, an aliquot of which was used for the determination of unreacted PC by the method of Ames[14].

Phosphorylcholine was estimated in the presence of the adsorbents. The Ames[14] method of phosphorus estimastion was used with the following modification required because of the presence of the adsorbents. The reaction zone was scraped off into a Corning glass tube and were well moistened with an appropriate volume (*ca.* 0.20–0.25 ml) of 10% $Mg(NO_3)_2$ in absolute alcohol. The tube was placed on a bath of boiling water for about 15 min to remove most of the solvents. The rest of the procedure followed the Ames[14] method, except that the adsorbents were removed by centrifugation before colour estimation. Where the amount of phosphorylcholine exceeded 0.1 μmole, larger volumes of 0.5 *N* HCl than that used by Ames[14] (1.5 ml) were used for hydrolysis of the pyrophosphate. The hydrolyzate was centrifuged and 1.5 ml of the clear supernatant were used for colour development.

The amount of phosphorylcholine produced was also determined in test-tubes according to the method of Ottolenghi[7].

RESULTS

Recovery of unreacted egg PC from the adsorbent

The results of the experiments in Table I show that the unreacted egg PC could almost completely (96–97%) be extracted by the method used, and deactivation of the enzyme can be affected by concentrated HCl or boiling.

Effect of substrate concentration on the degree of hydrolysis

Fig. 2 demonstrates the effect of substrate concentration on the extent of hydrolysis by 0.1 mg of enzyme (0.09 mg of enzyme protein) at 25°C in 1 h. The almost linear sharp initial rise in the curve denotes high conversions (*ca.* 80%) at low substrate to protein ratio up to a value of 1.3. Substrate saturation occurred when this ratio reached the value of 7.7. Thus, for the preparation of representative diglycerides from PC, it is profitable to use a substrate to protein ratio of about 1.3.

TABLE I

RECOVERY OF UNREACTED EGG PC FROM THE ADSORBENT

The lipids were extracted with three 3-ml portions of chloroform–methanol (1:1) containing 10% water (see text).

Expt. No.	*Materials applied in sequence on the TLC plate**	*Amount of egg PC recovered from the plate (μmole)***	*Recovery of egg PC (%)***
1	0.1 mg deactivated*** enzyme + 0.9 μmole egg PC	0.88 ± 0.01	97.0 ± 1.1
2	0.1 mg deactivated ‡ enzyme + 0.9 μmole egg PC	0.87 ± 0.03	96.0 ± 3.3
3	0.9 μmole egg PC	0.87 ± 0.03	96.0 ± 3.3

* Plates were kept at 25°C for 1 h after the addition of the reaction mixture.
** Average from four sets of experiments.
*** Deactivated by the addition of a drop of conc. HCl to the enzyme preparation.
‡ Deactivated on a bath of boiling water for 10 min.

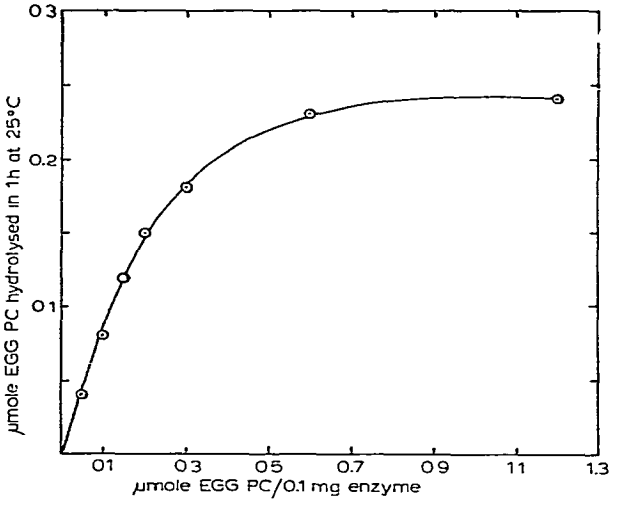

Fig. 2. Effect of substrate concentration on the extent of lipolysis. On each of seven sets (four in a set) of TLC plates was applied 0.1 mg of phospholipase C (0.09 mg of enzyme protein). On plates in sets 1–7 respectively, 0.05, 0.10, 0.15, 0.20, 0.30, 0.60 and 1.20 μmole of egg PC were applied. The reactions were allowed to proceed at 25°C for 1 h and the amounts of egg PC hydrolysed were estimated both from the amounts of unreacted PC and from the phosphorylcholine formed. The results from the two types of measurements were found to be identical within the limits of experimental error and the mean value has been plotted. Standard deviation: ±3%.

Time course of on-plate lipolysis

The time course of the on-plate reaction (Fig. 3) at 25°C was linear up to 35 min and the plateau region was reached within 1 h. This time course is almost coincident with that derived from measurements done in tubes[7,15]. In general, the re-

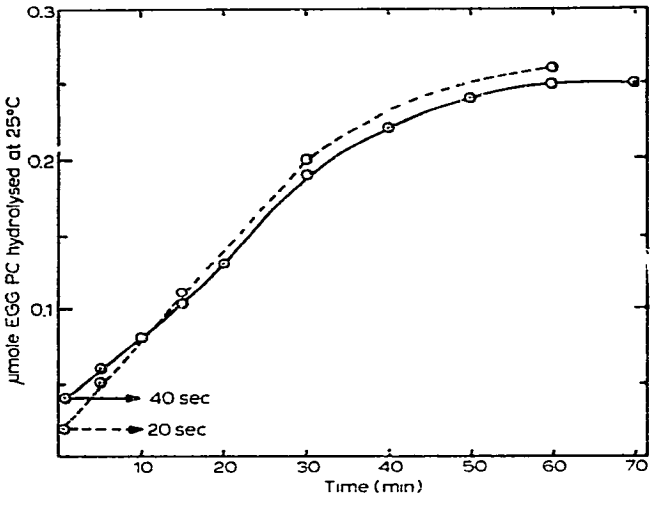

Fig. 3. Time course of lipolysis of egg PC by phospholipase C, carried out on a TLC plate (——) and in a tube (– – –). On each of ten sets (four in a set) of TLC plates was applied 0.1 mg of enzyme as a band over which 0.9 μmole of egg PC were added throughout the band as quickly as possible. The reactions at 25°C were allowed to proceed for 40 sec and 5, 10, 15, 20, 30, 40, 50, 60 and 70 min, and the amounts of egg PC hydrolysed were estimated both from the amounts of unreacted egg PC and from the phosphorylcholine formed. The results from the two types of measurements were found to be identical within the limits of experimental error and the mean values have been plotted. The estimations of similar lipolytic reactions at 25°C were done in tubes for 20 sec and 5, 10, 15, 30 and 60 min according to the method of Ottolenghi[7] and the results have also been plotted. Standard deviation: ±2%.

sults obtained by these two methods did not differ by more than 5%. Thus, the present method can profitably be used for the assay of phospholipase C.

A very interesting feature of both these time courses is that, even within 40 sec of the application of the substrate, 0.04 μmole (40 sec) and 0.02 μmole (20 sec) of egg PC were hydrolysed on the plate and in the tube respectively. No reaction products could be found either with deactivated enzyme or in the absence of enzyme, thus excluding decomposition of the substrate before addition of the enzyme. This suggests that the rate of this reaction over the first 40 sec is considerably higher than that for the rest of the time.

At 25°C the rate of conversion was low, 0.1 μmole (11.1%) in the first 15 min and 0.2 μmole (27.7%) in 1 h. Conversion data (not shown) taken at a few points on the time scale at 37°C show that 53.3% hydrolysis occurred within 15 min, and this temperature is recommended for the assay of the enzyme.

On-plate assay for phospholipase C

The following protocol is to be followed for this method.

A 0.1–0.2 mg amount of the enzyme protein in 0.05–0.1 ml of the enzyme solution and 1–2 μmole PC in 0.1 ml of diethyl ether–methanol (98:2) were applied on each plate and the reaction allowed to proceed in an ether-saturated chamber at 37°C for 15 min. After chromatographic resolution of the products, the activity of the enzyme may be estimated from the amount of either the phosphorylcholine formed or

the unreacted PC. For a control, the same reaction mixture but with deactivated enzyme is used.

DISCUSSION

This method provides an easy assay for phospholipase C, the substrate of which is insoluble in water. In the conventional methods[7,12] some means, usually vigorous shaking or sonication, must be employed to bring the substrate and enzyme into close contact. In the present on-plate method this is achieved by use of the large silica gel surface, as was the case in similar on-plate methods[8,9] developed by us. The advantage of the present assay over egg yolk turbidimetry[15] or the phosphorus-release method[7] is that here the unreacted substrate and all the reaction products are separated and the activity is measured from the phosphorus content of either the unreacted PC or the phosphorylcholine formed. When the phosphorus content of phosphorylcholine is to be estimated, the direct measurement in the presence of the adsorbents provides an extra advantage.

The present procedure is convenient for the preparation of representative diglycerides from PC. The reaction has to be carried out for 1 h at 25°C using higher amounts of both the substrate (*ca.* 4 μmole) and the enzyme (2 mg of our enzyme protein). The *sn*-1,2-diglycerides formed can be isolated by extracting the corresponding band, in a mini-column, with five 2-ml portions of diethyl ether. Such representative diglycerides have been used for the determination of the molecular species of PC obtained from different sources[2,3].

ACKNOWLEDGEMENTS

Thanks are due to the Council of Scientific and Industrial Research (C.S.I.R.), Government of India, for a Senior Research Fellowship to one of us (A.K.D.).

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