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Note

Micro-chromatographic test of transphosphatidylic activity of phospholipase D in algae and other plants

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While investigating the phospholipid composition of marine macrophytes we detected that chloroform–methanol extracts of some red algae contained phosphatidylmethanol (PM). The formation of PM during lipid extraction as a result of phospholipase D action is well known for higher plants[1–3]. However, the only alga in which phospholipase D had been found previously is a unicellular red alga *Porphyridium cruentum*[4]. We therefore decided to screen the phospholipase D distribution in marine macrophytes. This task required a micro-chromatographic procedure to test for the transphosphatidylic activity of phospholipase D. The technique developed, which is also applicable to other plants, showed that red algae and sea grasses contained phospholipase D, but the enzyme was not detected in all investigated species of green and brown algae.

EXPERIMENTAL

Lipids of different sources were extracted by modifications of the method of Bligh and Dyer[5]. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were isolated from hen egg yolk, diphosphatidylglycerol (DPG) was obtained from bovine heart and N-acylphosphatidylethanolamine (N-APE) from wheat flour. PM and phosphatidylglycerol (PG) were prepared from PC by the action of cabbage phospholipase D in the presence of methanol and glycerol, respectively[1,2]. A mixture of tritium-labelled phospholipids was kindly provided by Dr. V. P. Shevchenko (Institute of Molecular Genetics, Moscow). Lipids were purified by column and thin-layer chromatography (TLC). Micro-thin-layer plates were prepared as described earlier[6]. Molybdate reagent[7] and malachite green reagent[8] were used for the detection of phospholipids. Incubations of labelled lipids with enzyme extracts of algae in test-tubes or on thin-layer plates were performed as described[9].

Detection of transphosphatidylic activity of phospholipase D

Plant tissue (200 mg) was thoroughly ground with sand in a mortar. A 50% (v/v) solution of methanol (1 ml) was added and the mixture incubated for 15 min at room temperature. Then methanol (1.5 ml) and chloroform (1 ml) were added, the suspension was filtered and the residue washed with chloroform (1 ml) and reextract-

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ed with two portions (1 ml) of chloroform–methanol (1:1, v/v). After the addition of water (3 ml) and phase separation the chloroform layer was evaporated *in vacuo*, the lipids were dissolved in chloroform (0.5 ml). An aliquot (10–20 μl) of this solution was applied to a micro-thin-layer plate and chromatographed in chloroform–acetone–methanol–benzene–28% ammonium hydroxide (100:40:30:10:4). The spots of the phospholipids were located with molybdate reagent[7], then the chromatogram was treated with $HClO_4$ and sprayed with malachite green reagent[8].

Determination of transphosphatidylic activity of phospholipase D

Three aliquots (10–30 μl) of the lipid solution (volume taken is based on the results of the enzyme detection) were spotted in three lines (5–10 mm) on a micro-thin-layer plate and chromatographed as described above. The spots were located by charring after spraying with 10% (v/v) H_2SO_4 in methanol or with molybdate reagent[7]. The silica gel containing PM was then scraped off into a digestion tube and the silica gel with all the other phospholipids was collected in another test-tube. The phosphorus content was determined as described previously[7].

RESULTS AND DISCUSSION

The most sensitive procedures for determining phospholipase D activity utilize labelled substrates[3]. Usually the substrate is incubated with enzyme solution in a test-tube. We had previously elaborated a simple technique to screen phospholipase D in higher plants which included incubation of unlabelled substrate and enzyme solution on a chromatographic plate[9]. However, both *in vitro* and on-plate incubations failed to produce satisfactory results for algae despite of the fact that the substrates used in the experiments were highly labelled. The reason for this is the low hydrolase activity of algal phospholipase D.

Phospholipase D can be tested as phosphatidyltransferase[3]. A variant of this method was used by Comes and Kleinig[10] for enzyme determination in a slime mould *Physarum polycephalum* and by Roughan and Slack[11] in higher plants. Pieces of plant tissues were incubated with methanol, the lipids were extracted and the content of PM and other phospholipids was determined. Since it was difficult to fragment algae after addition of methanol, we ground the tissues before incubation. In doing so, an interesting result was obtained. Upon incubation with 20–100% methanol, homogenates of red algae produced PM. However, homogenates of higher plant tissues, *e.g.*, potato tubers and cabbage stumps, yielded PM with concentrated methanol, while others, such as roots of *Codonopsis lanceolata* containing phospholipase D of high activity[9], produced PM only when incubated with dilute methanol. Therefore, incubation with 50% methanol was chosen for our test, as at this concentration all plants, in which phospholipase D was detected by any technique, gave PM.

It had been demonstrated previously that PM formation proceeded rapidly if plant pieces were incubated with methanol[10,11]. We found that 15 min is sufficient in our procedure.

For estimation of phospholipase D activity, previous authors determined the phosphorus contents in PM and all other individual phospholipids separated by one-dimensional[11] or two-dimensional[10] TLC. We simplified this procedure by choosing an appropriate solvent system which separated PM from all major plant

phospholipids, such as PC, PE, PG and DPG, which remained in the zone with R_F less than 0.2. It was possible to determine the phosphorus content of the phospholipids in one test-tube without overloading the sample with silica gel. N-APE, occurring in some higher plants[12], moved ahead of PM.

Fig. 1 shows the separation of standard phospholipids and phospholipids from incubation mixtures. PM and other phospholipids can be resolved by more simple solvent systems, but our multicomponent system is superior as the results obtained are less dependent on factors such as air humidity, amount of silica gel, etc. This solvent system does not separate PM from some low polarity glycolipids, but glycolipids do not hinder PM detection by phospholipid sprays[7,8,13] or its quantifica-

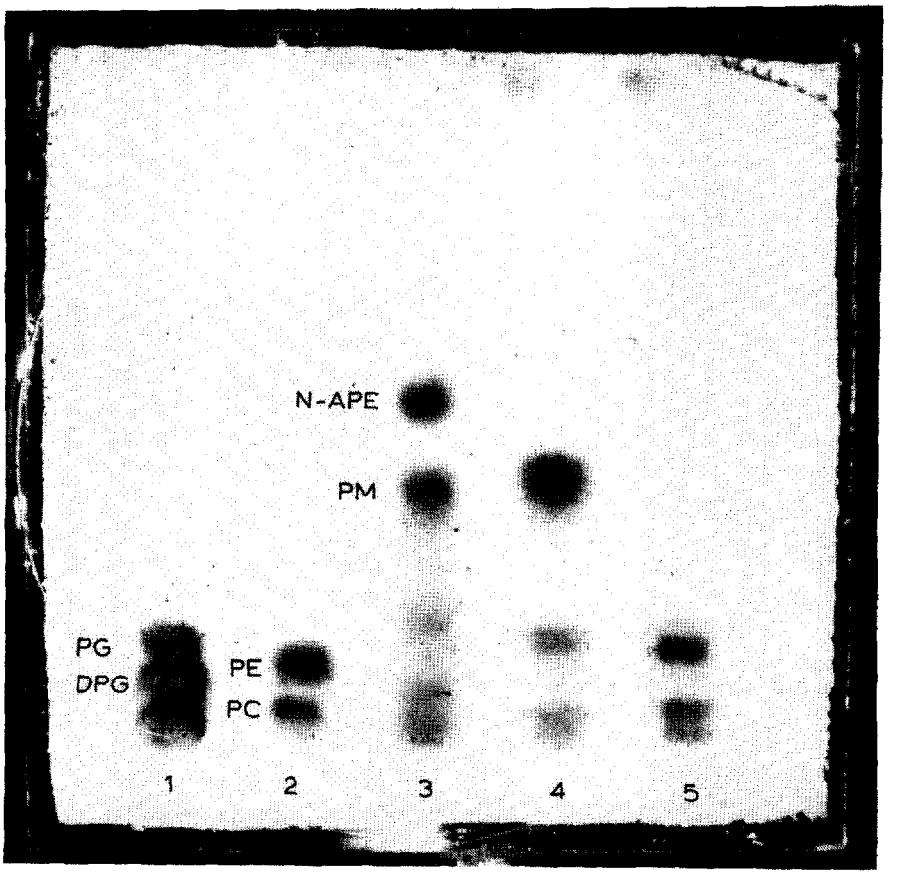

Fig. 1. One-dimensional chromatogram (6 × 6 cm) of standard phospholipids and phospholipids from mixtures of algal homogenates incubated with 50% methanol. Solvent system: chloroform–acetone–methanol–benzene–28% ammonium hydroxide (100:40:30:10:4 v/v). Spray reagent: malachite green[8]. 1 = Phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG); 2 = phosphatidylethanolamine (PE) and phosphatidylcholine (PC); 3 = N-acylphosphatidylethanolamine (N-APE) and phosphatidylmethanol (PM); 4 = phospholipids of red alga *Nemalion vermiculare* after incubation; 5 = phospholipids of brown alga *Laminaria cichorioides* after incubation. Just before the photograph was taken the plate was exposed to ammonia vapour for about 1 min. Green spots of phospholipids became blue while the orange background turned white. Some minutes later the plate became uniformly dark blue.

TABLE I

TRANSPHOSPHATIDYLIC ACTIVITY OF PHOSPHOLIPASE D OF SOME MARINE MACROPHYTES AND TERRESTRIAL HIGHER PLANTS

Enzyme activity is expressed as a percentage of conversion of phospholipids into phosphatidylmethanol during incubation of tissue homogenate in 50% methanol for 15 min. The results given are means ± S.D. from three determinations.

Marine macrophyte	*Enzyme activity*	*Higher plants**	*Enzyme activity*
Nemalion vermiculare	52.6 ± 3.5	*Asparagus schoberioides*	56.0 ± 2.3
Bossiella cretacea	11.4 ± 0.8	*Sanguisorba officinalis*	14.5 ± 3.1
Tichocarpus crinitus	6.2 ± 0.8	*Lespedeza bicolor*	15.7 ± 1.1
Ahnfeltia tobuchiensis	24.2 ± 2.8	*Euphorbia komaroviana*	31.7 ± 1.5
Mastocarpus ochotensis	11.4 ± 2.5	*Patrinia scabiosifolia*	28.9 ± 4.0
Zostera marina	4.4 ± 0.6	*Codonopsis lanceolata*	43.9 ± 1.5
Phyllospadix iwatensis	10.4 ± 1.1		

* Roots of *P. scabiosifolia* and leaves of the other plants were taken.

tion. The sensitivity of molybdate reagents[7,13] is insufficient for detection of PM in the case of low phospholipase D activity. Therefore we recommend the use of the more sensitive malachite green reagent after the molybdate spray.

The described technique was used to investigate phospholipase D activity in marine macrophytes. The enzyme was detected in none of the tested green algae (*Ulva fenestrata, Enteromorpha linza, Bryopsis plumosa, Codium yezoense*) and brown algae (*Leathesia difformis, Chordaria flagelliformis, Analipus japonicus, Chorda filum, Laminaria cichorioides, Dictyopteris divaricata, Sargassum pallidum, Pelvetia wrightii*). However, of seven species of red algae, only *Rhodomela larix* did not demonstrate activity. Low activity was also found in both sea grasses investigated.

Six species of higher plants in which phospholipase D had not been detected by our old technique[9] were then reinvestigated. The new method gave negative results for one sample only —leaves of *Lychnis fulgens*.

Table I shows the results of determination of transphosphatidylic acitivity of phospholipase D in red algae, sea grasses and terrestrial higher plants. We decided to express the enzyme activity as a relative concentration of PM. Although this index was influenced by many factors, such as the fragmentation of plant tissues, methanol concentration, phospholipid composition, etc., it clearly indicated the phospholipase D activity. Thus, we found PM in lipid extracts only of those algae which demonstrated enzyme activity in the test described, and the PM quantities in lipid extracts readily agreed with the index values obtained from the tests. Plants which were shown to be active using our old technique[9] gave high index values.

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