Purification of Phospholipase D from Citrus Callus Tissue

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Phospholipase D in extracts of soluble proteins from callus cultures derived from cotyledons of Citrus sinensis (L.) Osbeck is activated by Ca\textsuperscript{2+} and anionic detergents and has a pH optimum of 6.5. The enzyme was purified 703-fold over the crude protein extract with a yield of 15% by ammonium sulfate precipitation, ion exchange chromatography, gel filtration, hydrophobic interaction chromatography, and preparative acrylamide gel electrophoresis. Preparative electrophoresis was carried out using conventional slab gel equipment and electroelution of the sliced gel. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified phospholipase revealed two bands of the same staining intensity running at 94.2K and 90.5K.

Phospholipase D (EC 3.1.4.4) catalyzes the hydrolysis of the terminal phosphate diester bond in most diester glycerophospholipids. Primary short-chain alcohols can replace water in this reaction (1), leading to the formation of the corresponding phosphatidylalcohols (transphosphatidylation). The enzyme has been identified in tissues of a large number of plant species (2–4). The enzyme was purified to electrophoretic homogeneity from peanut seeds (5) and savoy cabbage leaves (6). The properties of phospholipase D from higher plants and other species have been reviewed in detail (3, 4).

In spite of its wide occurrence in the plant kingdom, the physiological role of phospholipase D is still unknown. Evidence for the participation of this enzyme in membrane phospholipid changes related to cold hardening and freeze injury (7–11) and water stress (12) has been reported. A high activity of phospholipase D has been found in storage tissues, especially in seeds (2). The breakdown of phospholipids during seedling growth has been attributed to the concomitant changes of phospholipase activity (13–15).

In the present study, the expression of phospholipase D in callus lines derived from Citrus sinensis is reported. The enzyme occurs in high activity and has the typical properties of phospholipase D from differentiated plant tissue. Therefore, callus cultures may serve as versatile models for studying the physiological function of plant phospholipases in vitro. In addition, a procedure for purification of the enzyme from this source was established.

MATERIALS AND METHODS

Tissue culture. Callus lines from explants of plants grown under aseptic conditions were established and subcultured essentially as described by Barthe et al. (16). Briefly, fertilized seeds of Citrus sinensis cv. Valencia from freshly harvested fruits were disinfected in 10% commercial bleach (5% sodium hypochlorite), washed three times in water, and placed on 0.8% agar for germination. When the plants were 3–6
cm tall, explants from cotyledons were transferred to Petri dishes containing callus medium (14) solidified by 0.8% agar. Upon callus formation, the isolated callus tissue was placed on fresh medium and subcultured every 5-6 weeks.

**Enzyme assay.** Twenty micromoles of DPPC containing 2.5 μCi 1,2-[14C]palmitoyl-sn-glycero-3-phosphocholine (NEN, Boston, MA) were emulsified in 2 ml water by sonication at room temperature (Micro-Ultrasonic Cell Disruptor, Kontes, Vineland, NJ). The standard enzyme assay mixture consisted of 20 μl (0.2 μmol) substrate, 50 mM CaCl₂, 1 mM SDS, 100 mM Mes/NaOH (pH 6.5), and 10 μl enzyme solution in a total volume of 100 μl. The reaction was initiated by addition of enzyme and allowed to continue at 30°C for 10-30 min. Incubation was stopped by addition of 5 μl of 5 M HCl and lipid extraction with chloroform/methanol as described recently (17) or with n-butanol. In the latter case, 100 μl butanol was added to the reaction vials and mixed by vortexing. Centrifugation at 1000g for 10 min facilitated phase separation. The butanol phase was removed, and the extraction was repeated in the same way. Finally, the butanol phases were combined. This procedure was used only for activity tests of crude enzyme preparations with high protein contents. DPPC, phosphatidic acid, and phosphatidylmethyl ether were separated by TLC on precoated silica gel plates (EM Science, Gibbstown, NJ) with chloroform/methanol/acetic acid/water (60/30/6/3, v/v). Silica gel containing radioactivity was scraped into chloroform/methanol/acetic acid/water (60/30/6/3, v/v) or chloroform/methanol/25% NH₃ (60/30/10, v/v). The suspension was chilled on ice for 10 min with occasional shaking before cell debris and PVPP were pelleted by centrifugation for 10 min at 1000g. The supernatant was centrifuged for 30 min at 20,000g to yield an almost clear supernatant (stage I of the purification).

An equal volume of saturated ammonium sulfate solution was added and the precipitated protein was centrifuged at 20,000g for 30 min after about 12 h. The pellets were resuspended in 200 ml Tris buffer by stirring for 2 h. Undissolved material was removed by centrifugation at 20,000g.

The clear, yellow supernatant (stage II) as applied to a column (2.5 × 40 cm) of QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) equilibrated with Tris buffer. The column was washed at 20 ml/h with the same buffer until no UV-absorbing material was detectable in the effluent. Bound protein was eluted by a NaCl gradient of 0 to 1 M NaCl in Tris buffer. Fractions of 20 ml were collected at a flow rate of 20 ml/h and assayed for phospholipase activity and absorbance at 280 nm. Fractions containing enzyme activity were pooled, and solid ammonium sulfate was added to achieve 70% saturation. After the pH was adjusted to 7.5, protein was allowed to precipitate for 16 h. It was collected by centrifugation at 20,000g for 30 min and resuspended by stirring for 2 h in 5 ml Tris buffer containing 0.5 M NaCl (Tris-NaCl buffer). Undissolved, dark brown material was removed by centrifugation at 40,000g for 30 min to yield a clear, nearly colorless supernatant (stage III).

A column (2.2 × 100 cm) of Sepharyl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) was packed and equilibrated with Tris-NaCl buffer as recommended by the manufacturer. The stage III enzyme solution was applied to the column at a flow rate of 4 ml/h. Elution was continued with the same rate while fractions of 5 ml were collected. Absorbance at 280 nm and phospholipase activity were measured in each fraction. The column was calibrated under the same separation conditions using a gel filtration molecular weight standard kit (Bio-Rad Laboratories, Richmond, CA), which comprised thyroglobulin (bovine), γ-globulin (bovine), ovalbumin (chicken), myoglobin (horse), and vitamin B-12.

The combined enzyme-containing fractions (stage IV) were subjected to hydrophobic interaction chromatography. A column (1.6 × 15 cm) packed with octyl-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) in Tris-NaCl buffer was used for this purpose. After sample application, the column was washed with the same buffer at 4 ml/h until no unbound material was detected in the effluent.
buffer without NaCl was then used for further elution at the same speed. Finally, bound material was detached from the column by elution with 0.2% (w/v) sodium cholate in Tris buffer. The eluate was collected in 5-ml fractions throughout the separation. Fractions with enzyme activity were combined and concentrated to 0.4-0.6 ml in an Amicon pressure cell equipped with a PM-30 filter (Amicon Co., Danvers, MA). The sample was washed twice in this way with Tris buffer and stirred overnight in concentrated form (about 0.5 ml) in the pressure cell with the filter in place. If necessary, precipitated material was pelleted by centrifugation in a tabletop centrifuge to obtain a clear supernatant (stage V).

The final purification of phospholipase D was achieved by preparative electrophoresis. A Laemmli system (18) was used with some modifications: SDS was omitted from all solutions; the pH of the separation gel buffer was 8.4; 100 μl of the stage V sample was mixed with 150 μl of a solution comprising 50% glycerol and 10% 2-mercaptoethanol; aliquots of 50 μl of this mixture were applied to adjacent slots in an LKB-2001 vertical slab gel electrophoresis unit (LKB Produkter AB, Bromma, Sweden) equipped with 1.5-mm combs; 8% gels were run at 2°C in the circulating cooling water; the separation was started at constant voltage (200 V) until the current dropped to 25 mA and then continued at constant current for about 4 h.

The gel was sliced with a razor blade into segments of 5 mm, or 2 mm in areas where enzyme activity was expected. The slices were transferred into sample cups of an Isco Electrophoretic Concentrator (Isco Inc., Lincoln, NE) for elution and concentration of protein bands. The concentrator contained Tris buffer (pH 8.4) in the electrode and inner chamber and the same buffer, diluted fourfold, in the sample cups. The electroelution was run for 2 h at 3 A. About 0.3 ml of concentrated protein solution was recovered per sample cup. Fractions with phospholipase activity were checked for purity by analytical SDS-PAGE and finally combined to obtain stage VI of the purification.

HPLC was used in another attempt to purify the phospholipase. An LDC/Milton Roy HPLC system (LDC/Milton Roy Analytical Instruments, Riviera Beach, FL) equipped with a TSK-gel column (0.75 X 7.5 cm), type DEAE-5PW (Phenomex, Palos Verdes, CA), was used. An aliquot of concentrated and desalted stage IV enzyme (200 μl, 240 μg protein) was injected and pumped through the column at 100 psi, 1 ml/min. The phospholipase activity was recovered by elution with a linear gradient of 0-0.5 M NaCl in Tris buffer (pH 8.0). Fractions of 2 ml were collected, assayed for enzyme activity, and analyzed by SDS-PAGE.

Analytical methods. Protein was determined by the Coomassie binding assay of Bradford (19). Analytical SDS–PAGE was conducted as described by Laemmli (18) with slight modifications. Protein bands were detected by staining with Coomassie or by silver staining using a Bio-Rad kit. Ultrathin-layer isoelectric focusing of purified samples of phospholipase D was performed on precoated gels (Servalyt Precotes, pH 3-6, Serva Fine Biochemicals Inc., Westbury, NY) as recommended by the manufacturer.

RESULTS

Purification

Mechanical disruption of frozen callus tissue and centrifugation resulted in a clear supernatant devoid of most cellular membrane systems, which contained phospholipase D in rather high activity. The specific activity of 0.66 ± 0.05 μmol min⁻¹ mg protein⁻¹ (±SD, n = 4) is comparable to that of extracts of soluble proteins from differentiated tissues of higher plants (2).

Only a moderate purification of 43-fold was achieved by application of standard procedures: ammonium sulfate precipitation, ion exchange chromatography (Fig. 1), and gel filtration (Fig. 2). Analytical SDS–PAGE of this preparation revealed a major band without activity (see below) occurring at 26.5K and several minor bands, most of them of higher molecular weight (Fig. 3, lane d). The next step, hydrophobic interaction chromatography (Fig. 4), did not eliminate the 26.5K band, but several of the minor bands without phospholipase activity were removed. The enzyme activity was bound almost com-

![Fig. 1. QAE-Sephadex ion exchange chromatography of phospholipase D. The separation was conducted as described under Materials and Methods. Elution with the NaCl gradient started at fraction 32. Absorbance at 280 nm (○) and phospholipase activity (▲) were measured in each fraction.](image-url)
FIG. 2. Gel filtration of phospholipase D with Sephacryl S-300. Details of the separation are given under Materials and Methods. The symbols are the same as those used in Fig. 1.

cepted, because it was necessary to eliminate most of the minor bands to obtain an electrophoretically homogeneous preparation in the next step, preparative slab gel electrophoresis. Figure 5 shows that the main contaminating band, i.e., the 26.5k band mentioned above, and other minor contaminations were clearly separated by this procedure from gel areas, which contained the highest phospholipase activity. The enzyme could be eluted from the sliced gel in very high yields by electroconcentration, provided that the pH of the separation gel buffer was carefully controlled. Rapid inactivation was observed above pH 8.4.

HPLC conducted as described under Materials and Methods could not replace the octyl-Sepharose step or the preparative electrophoresis. The activity was recovered during elution with the salt gradient, but only a few contaminating bands were eliminated by this procedure (not shown).

Table I summarizes the results of a complete purification. An overall 703-fold increase of specific activity with a yield of 15% was achieved.

Properties

Phospholipase D from citrus callus tissue showed properties very similar to those of the same enzyme from other plant sources with respect to cofactor require-
TABLE I
PURIFICATION OF PHOSPHOLIPASE D FROM CITRUS CALLUS

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total protein (mg)</th>
<th>Total activity (µmol min⁻¹)</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>510.0</td>
<td>332.0</td>
<td>0.66</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>II</td>
<td>155.0</td>
<td>143.0</td>
<td>0.92</td>
<td>42.9</td>
<td>1.4</td>
</tr>
<tr>
<td>III</td>
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<td>252.0</td>
<td>13.0</td>
<td>75.8</td>
<td>19.7</td>
</tr>
<tr>
<td>IV</td>
<td>8.71</td>
<td>106.0</td>
<td>13.6</td>
<td>31.9</td>
<td>43.3</td>
</tr>
<tr>
<td>V</td>
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<td>50.3</td>
<td>68.0</td>
<td>15.1</td>
<td>103.0</td>
</tr>
<tr>
<td>VI</td>
<td>0.11</td>
<td>49.9</td>
<td>464.0</td>
<td>15.0</td>
<td>703.0</td>
</tr>
</tbody>
</table>

Stages of purification: I, crude extract; II, ammonium sulfate precipitation; III, ion exchange chromatography; IV, gel filtration; V, hydrophobic interaction chromatography; VI, preparative electrophoresis.

The phospholipase activity is activated by Ca²⁺; the maximal effect was observed at 50 mM CaCl₂. About 25% of the optimal activity was insensitive to CaCl₂ (Fig. 6), and 4% of the activity remained in the presence of 5 mM EGTA (not shown). SDS was used as an activator in this study. A concentration of 1 mM at a substrate concentration of 2 mM was sufficient for maximal activation (Fig. 7). A pH optimum of 6.5 was found in the presence of 1 mM SDS and 50 mM CaCl₂ (Fig. 8).

Both the crude protein extract and the purified enzyme from citrus callus catalyze the hydrolysis to phosphatidic acid, as well as the transphosphatidyltransfer reaction, when methanol or ethanol at concentrations of 10 or 20% was added to the reaction mixture (not shown). This is another characteristic feature of phospholipase D from plant sources (1, 3, 4).

The activity of the purified phospholipase was unstable. About 95% of the activity was lost when stored in Tris buffer containing 2.5 mM DTT for 18 days at 0–4°C and 99% at −15°C.

SDS-PAGE of the purified enzyme revealed two bands of roughly the same staining intensity running closely to...
FIG. 7. Activation of phospholipase D by SDS. The SDS concentration in standard assays was varied for determination of phospholipase activity using a stage IV enzyme preparation.

Together at apparent molecular weights of 94.2 ± 0.7K and 90.5 ± 0.8K (±SD, n = 7). These bands were observed in separations on 8 or 12% gels (Fig. 3, lane f), on gels containing 3.6 M urea, or in separations run without SDS (Fig. 5). They were detected equally well by staining with Coomassie or silver. They were also visible as minor bands in crude protein extracts (Fig. 3, lanes a and b). The apparent molecular weight determined by SDS-PAGE corresponds reasonably well to the result of the gel filtration step: the phospholipase D activity eluted at an apparent molecular weight of 90.5K from the Sephacryl S-300 column calibrated with molecular weight standard proteins. The isoelectric point of phospholipase D determined by isoelectric focusing was 5.0 (gel not shown).

DISCUSSION

The procedure established here to purify phospholipase D from citrus callus tissue resulted in a reasonable increase in specific activity (Table I), comparable to the 680-fold purification reported for the isolation of the enzyme from cabbage leaves (6). The increase in total activity in stage III compared with stage II may be due to the removal of endogenous inhibitors. A similar rise in activity during the first steps of purification has been observed before, and also has been explained by the presence of soluble inhibitory factors (1). The rather low yield is probably due to the instability of the purified enzyme. The crucial purification step was the preparative acrylamide slab gel electrophoresis and subsequent electroelution (Fig. 5). Conventional means of protein purification resulted in only a moderate increase in specific activity and failed to achieve complete purification. Preparative HPLC has been used for purification of plant enzymes in a similar situation, e.g., Geoffroy et al. (20). The results presented here suggest that electrophoresis with standard equipment can be an alternative when appropriate HPLC columns or equipment suitable for protein work are not available.

The enzymatic properties of phospholipase D from many higher plants have been analyzed and found to be very similar (3, 4). The enzyme from citrus callus fits into this scheme with respect to pH optimum, activation by Ca²⁺ and anionic detergents, and the ability to catalyze the transphosphatidylation reaction in the presence of primary alcohols.

In contrast, purification of phospholipase D from different sources revealed high variability of its molecular properties. Maximal molecular weights of 200,000 determined by gel filtration and 112,500 measured by SDS-PAGE have

FIG. 8. Effect of pH on the phospholipase D activity. Sodium acetate (pH 4.5–6), Mes/NaOH (pH 6.5–7), and Tris (pH 7.5–8.5) were used as buffer substances.
been reported for the enzymes isolated from peanut seed (5) and savoy cabbage leaves (6).

These may be oligomeric proteins, since much smaller molecular weights (22K and 48.5K) were found by electrophoresis in the presence of urea (6) or by sedimentation equilibrium centrifugation (5).

An apparent molecular weight of 90.5K was determined by gel filtration for phospholipase D from citrus callus. SDS-PAGE under different conditions revealed two bands of the same staining intensity running at 94.2K and 90.5K, but no indications for fragmentation into smaller units were observed, not even in the presence of 3.6 M urea.

The preparative purification procedures failed to separate both bands and, therefore, it is not possible to decide whether both bands are catalytically active. At least, both polypeptides have very similar properties; they copurified during a five-step procedure without a change in their relative concentration. They may represent two forms of the same protein differing in a post-translational modification like covalent binding of carbohydrate or partial proteolytic degradation.

Callus cultures could serve as a simple and versatile model to study the molecular properties of phospholipase D and some aspects of the physiological role of this enzyme in vitro.

REFERENCES


