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Multiple Forms of Phospholipase D following Germination and during Leaf Development of Castor Bean[1]

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Multiple molecular forms of phospholipase D (PLD; EC 3.1.4.4) were identified and partially characterized in endosperm of germinated seeds and leaves of castor bean (*Ricinus communis* L. var Hale). The different PLD forms were resolved by nondenaturing polyacrylamide gel electrophoresis, isoelectric focusing, and size-exclusion chromatography. PLD was detected with both a PLD activity assay and immunoblots with PLD-specific antibodies. There were three major forms of PLD, designated types 1, 2, and 3, based on their mobility during nondenaturing polyacrylamide gel electrophoresis. Molecular masses of the PLD variants were estimated at 330, 230, and 270 kD for the types 1, 2, and 3, respectively. Isoelectric points of the native type 1, 2, and 3 PLDs were approximately 6.2, 4.9, and 4.8. Under the in vitro assay conditions used, the three forms of PLD exhibited the same substrate specificity, hydrolyzing phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) but not phosphatidylserine (PS) and phosphatidylinositol (PI). The three forms of PLD differed in their substrate preferences, and the order of activities was: PLD 1, PE > PG = PC; PLD 2, PE > PG > PC; PLD 3, PE = PG = PC. The K_m values of PLDs 1, 2, and 3 for PC were 1.92, 2.62, and 5.18 mM, respectively. These PLDs were expressed differentially following seed germination and during leaf development. Type 1 was found in the early stages of seedling growth and in young leaves, type 2 was present in all the tissues and growth stages examined, and type 3 was expressed in senescent tissues. The PLDs shifted from largely cytosolic to predominantly membrane-associated forms during leaf development. The present studies demonstrate the structural heterogeneity of plant PLD and growth stage-specific expression of different molecular forms. The possible role for the occurrence of multiple molecular forms of PLD in cellular metabolism is discussed.

PLD (EC 3.1.4.4), which hydrolyzes phospholipids to generate PA, is often the most abundant phospholipase found in plant tissues. Although some studies have been carried out on PLD describing its catalytic kinetics, activity modulation, and substrate specificity (Heller, 1978; Wang, 1993, and refs. therein), little is known regarding its structural heterogeneity. The possibility of the presence of more than one form of PLD has been suggested in some studies of the biochemical properties of this enzyme. Early reports indicated that the soluble and membrane-associated PLD from cabbage leaves had different substrate preferences, with the former hydrolyzing choline plasmalogen and the latter hydrolyzing ethanolamine plasmalogen (Lands and Hart, 1965; Slotboom et al., 1967). The reported molecular masses of soluble PLD isolated from several plant species varied from 71 to 200 kD (Heller et al., 1974; Allgyer and Wells, 1979; Witt et al., 1987; Lee, 1989a). It is not clear whether the variability arises from differences in species or in specific tissues used for purification. Recent studies in animals have suggested the presence of isotypes of PLD based on the differences in substrate specificity, cofactor requirement, and subcellular location (Wang et al., 1991; Huang et al., 1992). Evidence for the presence of multiple forms of PLD, however, has not been presented.

Understanding the molecular multiplicity of PLD is of particular importance in studies of the regulation and function of PLD in cellular metabolism. This is because PLD activity has been implicated in diverse cellular processes such as membrane deterioration during senescence and stress injuries (McCormac et al., 1993; Voisine et al., 1993), storage lipid mobilization following seed germination (Herman and Chrispeels, 1980), and mediation of cellular responses to hormonal and environmental stimuli (Di Nola and Mayer, 1986; Lee, 1989b; Acharya et al., 1991). Recent studies in animals have pointed to PLD as a critical component in signal transduction pathways (Billah, 1993, and refs. therein). The presence of at least two PLD isoforms, one PC selective and another PI selective, has been suggested, and each of them is activated by distinct mechanisms in cell stimulation (Huang et al., 1992). For PLC and PLA_2, isoforms and multiple genes have been identified and isolated from mammalian tissues, and the molecular heterogeneity plays a vital role in their cellular functions (Dennis et al., 1991). In the case of plant PLC, there are at least two forms that exhibit different substrate specificities and subcellular locations (Yotsushima et al., 1993). An important question in understanding the role of PLD in plant metabolism is, therefore, whether or not different forms of PLD are involved in phospholipid breakdown in different cellular processes such as membrane deterioration under stress and senescence, lipid turnover during normal growth and development, and transmembrane-signaling pathways.

We recently reported the purification of soluble PLD from

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Abbreviations: g_{av}, average gravity; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEOH, phosphatidylethanol; PG, phosphatidylglycerol; PI, phosphatidylinositol; pI, isoelectric point; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine; PVDF, polyvinylidine difluoride.

endosperm of germinated castor bean (*Ricinus communis* L.) and immunological detection of PLD in several plant species (Wang et al., 1993). PLDs from several dicot and one monocot species cross-reacted with polyclonal antibody raised against purified castor bean PLD and exhibited similar molecular weights under denaturing conditions. Those results indicate that PLDs of several plants are structurally related. On the other hand, castor bean PLD was resolved into more than one activity peak on hydrophobic interaction chromatography and nondenaturing PAGE, which raised the question of whether different molecular forms of PLD occur in the same tissue. The present study provides evidence for the presence of multiple molecular forms of PLD in castor bean and shows that expression of these PLDs is well regulated after castor bean germination and during leaf development.

MATERIALS AND METHODS

Materials

Coatless seeds of castor bean (*Ricinus communis* L. var Hale) were germinated at 30°C in the dark up to 5 d (Wang and Moore, 1990). Endosperm was harvested for protein extraction and activity assay. Castor bean leaves were sampled from plants grown under cool-white fluorescent lights at 22°C. Polyclonal antibodies were raised in rabbits against a purified 92-kD protein with PLD activity from endosperm of d-2 germinated seeds (Wang et al., 1993). Radioisotopes, chemicals, and other reagents were obtained from the sources previously reported (Wang et al., 1993).

Tissue Fractionation and Isolation of PLD Variants

Endosperm halves of germinated castor bean (0–5 d) or leaves from 8-week-old plants were harvested separately and ground to fine powder in liquid N_2 with a mortar and pestle. All the following steps were carried out at 4°C unless stated otherwise. Proteins were extracted with buffer A containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 500 mM Suc, 0.5 mM PMSF, and 2 mM DTT. The homogenate was filtered through three layers of cheesecloth and centrifuged at 10,000*g* for 20 min. After the fat layer was removed, the supernatant was centrifuged at 110,000g_{av} for 60 min. The resultant supernatant (cytosolic fraction) and pellet (microsomal fraction) were suspended in buffer B (the same as buffer A except Suc was omitted). Portions of the cytosolic proteins were precipitated by adding slowly an equal volume of −20°C acetone with vigorous stirring. The precipitated proteins were pelleted at 12,000*g* for 20 min. Pelleted proteins were dissolved in buffer B, and undissolved materials were removed by centrifugation.

To isolate PLD variants, the acetone-precipitated proteins were adjusted to 5 mM DTT and 5% (v/v) glycerol prior to loading onto a nondenaturing polyacrylamide gel. The gel consisted of 8% (w/v) acrylamide (pH 8.8) in the resolving phase and 3.5% (pH 6.8) in the stacking phase. The gel was run at a constant voltage of 80 V for 30 min, and the voltage was then increased to 120 V at 4°C. After electrophoresis, the gel was sliced and placed directly in reaction mixtures for assaying PLD activity. The reactions were initiated by adding dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]choline as substrate. The components in the reaction mixture, substrate preparation, and assay conditions are described below. Proteins in the slices containing PLD activity were electroeluted with a vertical electroelutor according to the manufacturer's instruction (Bio-Rad). The electroelution was run for 4 h at 9 mA/tube. The eluted samples were assayed for PLD activity and checked for purity by SDS-PAGE. The isolated PLDs 1, 2, and 3 were either used for characterization or stored at −80°C until use.

PLD Activity Assay

PLD activity was assayed by using either 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-glycero-3-P-choline or dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]choline (Amersham) as substrates, depending on the experiment. The acyl-labeled PC was used for assaying both hydrolysis and transphosphatidylation reactions of PLD. The formation of phosphatidylalcohol serves as a better indicator of PLD activity than formation of PA, the hydrolytic product of PLD, since the former is not readily metabolized further. Choline-labeled PC was used for quick screening of fractions containing PLD activity. In both cases, 2.5 μCi of radioactive PC (500 μCi/mmol) was mixed with 20 μmol of cold PC (egg yolk; Sigma) in chloroform, and the mixture was dried under a stream of N_2. The lipid was emulsified in 1 mL of H_2O by sonication at room temperature. A standard enzyme assay mixture contained 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, 20 μL of substrate (0.4 μmol), and 20 μL of enzyme solution in a total volume of 200 μL. The assay conditions and reaction product separation and quantitation were detailed elsewhere (Wang et al., 1993).

In substrate-specificity experiments, nonradioactive phospholipids, PC, PE, and PG were derived from egg yolk, PI was from soybean, and PS was from bovine brain (Sigma). The fatty acid composition of these lipids was: PC and PG, 34.7% palmitate, 12.8% stearate, 30% oleate, 14.9% linoleate, and 3.3% arachidonate; PE, 21.6% palmitate, 2.2% stearate, 23.9% oleate, 14.9% linoleate, 8.3% arachidonate, and 1.5% behenate; PI, 36.6% palmitate, 9.1% stearate, 6.3% oleate, 41.7% linoleate, and 3.8% linolenate; PS, 44.6% stearate and 48.9% oleate (data obtained from Sigma). The substrate preparation, reaction conditions, lipid extraction, TLC separation, and lipid identification were the same as described above. PA and PEOH spots were scraped, and the amounts were quantitated by measuring phosphorus content as described (Rouser et al., 1970).

Electrophoresis and Immunoblotting

For immunoblot analysis of PLD resolved on nondenaturing PAGE, the gel after electrophoresis was immersed in 0.05% SDS in a protein transfer buffer (12.5 mM Tris and 100 mM Gly) for 10 min. The SDS-soaked gel was briefly rinsed with the transfer buffer. Subsequent procedures for transferring the proteins onto PVDF membranes were the same as previously reported for immunoblotting of SDS-PAGE gels (Wang et al., 1993). Briefly, proteins were electroblotted onto a PVDF membrane. The blot was then incubated with PLD antisera (1:1000 dilution) in PBS containing 5%

(w/v) nonfat dry milk. The antigen-antibody complexes were made visible with alkaline phosphatase conjugated with goat antibody against rabbit immunoglobulin.

Nondenaturing IEF was performed on a minigel system according to a published procedure (Robertson et al., 1987) with the following modifications: minigels were 0.75 mm thick and composed of 3.5%, pH 5 to 8, and 1.5%, pH 3 to 10, ampholytes. Protein (70 μg) was mixed with an equal volume of 50% (v/v) glycerol and 4% ampholytes in the same pH range used to prepare the gel. After electrophoresis PLD resolved on IEF PAGE was both assayed for activity and immunoblotted as described for nondenaturing PAGE.

In two-dimensional PAGE analysis of PLD, proteins (15 μg) were mixed with an equal volume of sample buffer (9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, and 1.6% [pH 5–7] and 0.4% [pH 3–10] ampholytes). The first-dimensional gel (9.2 M urea, 4% acrylamide, 2% Triton X-100, and ampholytes in the same concentrations as in the sample buffer) was prefocused at 200 V for 5 min, 300 V for 7 min, and 400 V for 7 min, after which the sample was loaded, covered with overlay buffer (9 M urea, 0.8% [pH 5–7] and 0.2% [pH 3–10] ampholytes, and bromphenol blue), and electrophoresed at 500 V for 10 min and then 750 V for 3.5 h at room temperature. The anode buffer was 10 mM phosphoric acid and the cathode buffer was 10 mM NaOH, both of which had been thoroughly degassed. The first-dimensional gels were then incubated in equilibration buffer (0.0625 M Tris-HCl, pH 6.8, 2.3% [w/v] SDS, 5% 2-mercaptoethanol, 10% glycerol, and bromphenol blue) for 2 min and loaded onto a slab SDS-PAGE (3.5% stacking, 8% resolving). After the SDS-PAGE, resolved PLD proteins were transferred onto PVDF membranes and visualized by immunoblot with PLD antibodies.

Size-Exclusion Chromatography

Size-exclusion chromatography was performed on a column (1.5 × 80 cm) containing Bio-Gel 50 (Bio-Rad). The column was equilibrated with a Tris-Gly PAGE buffer (25 mM Tris and 200 mM Gly) plus 2 mM DTT. The flow rate was 0.75 mL/min, which was controlled by an Econo chromatography system (Bio-Rad). Fractions (0.5 mL) were collected and assayed for PLD activity and for the presence of PLD proteins. Size standard proteins were carbonic anhydrase, 29 kD; alcohol dehydrogenase, 150 kD; β-amylase, 200 kD; apoferritin, 443 kD; and thyroglobulin, 669 kD (Sigma).

Protein Assay

Protein content was determined with a dye-binding assay according to the manufacturer's instructions (Bio-Rad).

RESULTS

Multiple Molecular Forms of PLD Resolved on Nondenaturing PAGE

The initial purification of PLD from castor bean involved the use of endosperm after 2 d from the start of imbibition (Wang et al., 1993). The highly purified PLD was found to be composed of a single 92-kD protein. Polyclonal antibodies were prepared against the 92-kD protein resolved on SDS-PAGE. The antisera, which were specific to PLD and were able to precipitate PLD from crude protein extract of castor bean (Wang et al., 1993), were used as a probe to investigate the expression of PLD in castor bean.

To enhance PLD concentrations in endosperm protein extracts, soluble PLDs were concentrated by acetone precipitation of the 110,000g_{av} supernatant fraction of endosperm protein extracts. This step retained more than 50% of PLD activity and it removed 90% of total soluble protein, resulting in a more than 4-fold enrichment of PLD-specific activity and enhanced immunodetection of PLD proteins resolved on nondenaturing PAGE. When the proteins were separated on nondenaturing PAGE, measurement of PLD activity in the gel slices revealed different patterns between PLDs extracted from the endosperm of 2- and 5-d germinated seeds. PLD activities in 2-d endosperm extracts were resolved into two distinct peaks: one migrated at 12 mm and another at 21 mm of the gel (Fig. 1A). By contrast, the PLD profile from 5-d endosperm extracts showed only one PLD activity peak spread between 21 and 24 mm (Fig. 1A). The minor PLD activity between the 12- and 21-mm PLD peaks of 2-d endosperm is due to the residual vertical streaking effect of proteins in the gel, which was noticeable when an immunoblot of the gel was overdeveloped.

To determine PLD-banding patterns resolved by nondenaturing PAGE, a parallel gel was run and the resolved

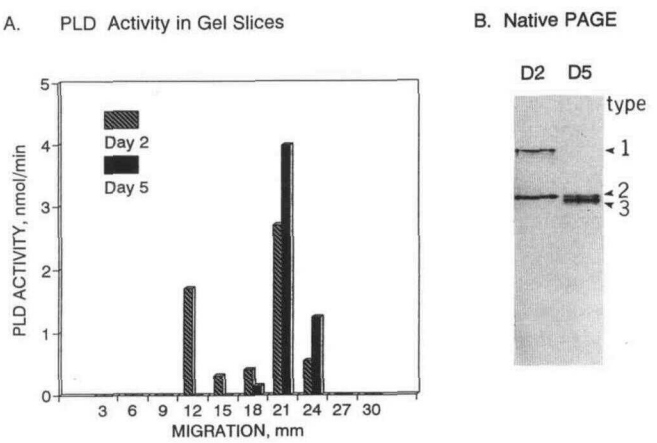

Figure 1. Nondenaturing PAGE (8%) separation of PLDs extracted from 2- and 5-d incubated endosperm of castor bean. Soluble PLDs (110,000g_{av} supernatant) from endosperm were precipitated with chilled acetone prior to PAGE analysis. The same amounts of proteins (65 μg/lane) were loaded, and the sample preparations and PAGE running conditions were as described in the text. A, PLD activity profile assayed in gel slices (3 mm/slice). The assay mixtures consisted of 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, and 2 mM PC (egg yolk), and activity was measured based on the release of choline from dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]-choline. B, Immunoblot of the nondenaturing PAGE gel with PLD antibodies. PLD antibodies (1:1000 dilution) were used and the blots were made visible using alkaline phosphatase conjugated to goat antibodies against rabbit immunoglobulin. The positions of the PLD activity in the gel matched those on the immunoblot. Type 1, 2, and 3 PLDs are designated according to their mobility.

proteins were transferred onto a PVDF membrane and immunoblotted with antibodies against the 92-kD PLD. The immunoblot showed two distinct bands of PLD proteins in 2-d endosperm (Fig. 1B), and their positions matched those of the PLD activity peaks. PLD from 5-d endosperm extracts was, however, resolved into two clustered protein bands; the major PLD band migrated slightly faster than the band that had the same mobility as the fast-moving PLD in 2-d endosperm. The differences between the immunoblot and PLD activity measurement of gel slices of PLDs from 5-d endosperm resulted from the differences in the degree of resolution by the two methods, and the two PLD bands on the immunoblot were too close to be resolved by slicing. To simplify the description of the PLD variants, the PLDs with the same slow and fast migration rate as that from the 2-d endosperm on the nondenaturing gel were designated types 1 and 2, respectively, and the PLD with the same migration rate as the major PLD of 5-d endosperm was referred to as type 3 (Fig. 1B).

Separation of Multiple Forms of PLD on IEF and Size-Exclusion Chromatography

The molecular forms of PLD resolved on nondenaturing PAGE could differ in their molecular mass, charge, conformation, or a combination of these. Therefore, the multiplicity of PLDs was further analyzed, and their pIs and molecular masses were compared under nondenaturing conditions. The differences in pIs of the PLD variants were assessed using a vertical slab IEF-PAGE system (Robertson et al., 1987), and the presence of PLDs was made visible by immunoblotting and PLD activity assays. When crude supernatant or acetone-precipitated proteins were used, PLDs from endosperm of 2-d germinated seeds were separated into two protein bands whose pIs were estimated at 6.2 and 4.9, respectively (Fig. 2). PLD from 5-d endosperm gave two close bands at pIs of approximately 4.8 to 4.9. The presence of PLD in those bands was confirmed because the corresponding immunoreactive PLD protein bands contained PLD activity. IEF analysis of the isolated PLDs indicated that the PLD band with a pI of 6.2 was derived from PLD 1, and the PLDs with pIs of 4.9 and 4.8 were from type 2 and 3 PLDs of 2-d and 5-d endosperm, respectively. The band intensity of PLD 2 relative to PLD 1 on the IEF-PAGE immunoblot of 2-d endosperm (Fig. 2) is much weaker than that shown in Figure 1B. This result was likely due to the differences among the PLD variants in their efficiencies of binding the gel matrix and electrotransferring onto the PVDF membranes in the two experimental methods.

The native molecular masses of the PLD forms were estimated by size-exclusion chromatography. Acetone-enriched PLD from endosperm of 2-d germinated seeds was separated into several overlapping peaks at molecular masses ranging from approximately 230 to 330 ± 40 kD (Fig. 3). The major PLD peak of 5-d endosperm extracts was estimated at 270 ± 40 kD. Proteins from the 230- and 330-kD fractions of 2-d endosperm extracts were concentrated, separated on nondenaturing PAGE, and then immunoblotted. The 330-kD activity peak gave a PLD protein band corresponding to type 1

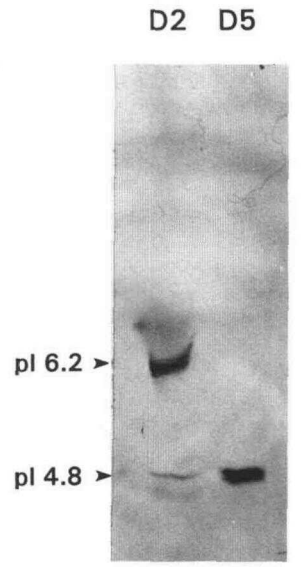

Figure 2. PLDs from 2- and 5-d endosperm resolved by nondenaturing IEF. Acetone-precipitated proteins (65 μg/lane) were separated on nondenaturing IEF (pH 3–10). To make PLD protein visible by immunoblot, the IEF gel was incubated in 0.05% SDS for 10 min prior to transferring onto a PVDF membrane.

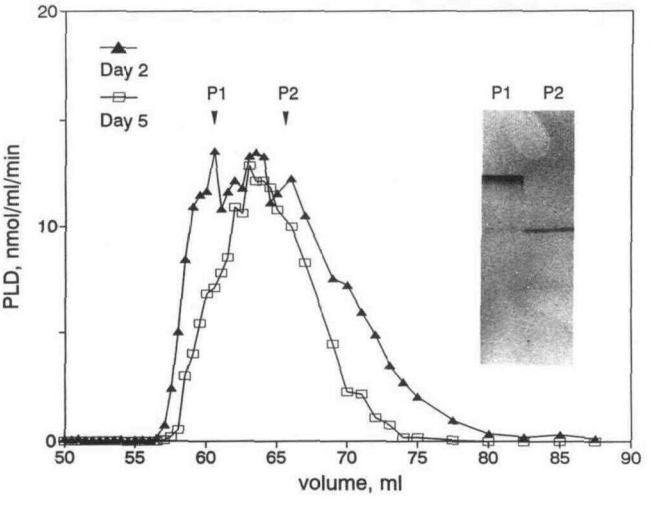

Figure 3. Activity and protein (inset) profiles of PLDs from 2- and 5-d endosperm resolved by size-exclusion chromatography (Bio-Gel 50, Bio-Rad). Acetone-precipitated protein extracts (3 mg) were loaded onto a column (1.5 × 80 cm). Flow rate was 0.75/min, and 0.5 mL/fraction was collected. PLD activity was measured by assaying the release of [^{3}H]choline from dipalmitoyl-3-glycero-P-[*methyl*-^{3}H]choline. Inset, Immunoblot of the PLD proteins resolved by nondenaturing PAGE. Proteins were collected from the two activity peaks, P1 and P2.

PLD and the 230-kD peak corresponded to a protein with the same mobility as that of type 2 PLD (Fig. 3, inset).

Analysis of PLD Variants on Denaturing PAGE

To gain insights into the molecular composition and catalytic properties of these PLD variants, the individual PLDs 1, 2, and 3 were partially purified for comparative studies. Type 1 and 2 PLDs from 2-d endosperm were electroeluted from the gel slices of nondenaturing PAGE, which clearly separated the two variants. Type 3 PLD was electroeluted from nondenaturing PAGE of 5-d endosperm. Distinct separation of type 3 from type 2 PLD was difficult because of the closeness of the type 2 and 3 bands on the gel. Based on the band intensity on the immunoblot of the nondenaturing PAGE gel, the isolated PLD 3 from 5-d endosperm contained less than 5% of PLD 2 (data not shown). The specific activities of PLDs 1, 2, and 3 were enhanced approximately 147-, 59-, and 135-fold, respectively, from 110,000g_{av} supernatant (Table I). The eluted proteins were subjected to SDS-PAGE, and Coomassie blue staining of the eluted proteins revealed multiple protein bands in these fractions (data not shown), indicating that the PLDs 1, 2, and 3 were only partially purified. When the denatured PLDs from SDS-PAGE were immunoblotted with PLD antibodies, type 1, 2, and 3 PLDs all showed a very similar molecular mass at 92 kD (Fig. 4A). The isolated PLDs 1, 2, and 3 were further subjected to two-dimensional PAGE analysis. The first-dimensional IEF contained 9.2 M urea, and under such denaturing conditions all three molecular forms appeared as a 92-kD protein with a pI at 5.6 (Fig. 4B). These results indicate that these PLD variants contain the same or very similar 92-kD proteins and that the multiple forms resolved under nondenaturing conditions did not result from proteolytic cleavage.

Catalytic Properties of PLD Variants

To test whether these PLD forms were biochemically distinguishable, the isolated PLDs 1, 2, and 3 were compared for the effects of pH, Ca^{2+}, and SDS on their activities, catalytic constants, substrate specificity and preference toward head groups of phospholipids, and relative hydrolysis and transphosphatidylation activities. The three PLD variants exhibited similar activity dependence on pH, Ca^{2+}, and SDS under the in vitro assay conditions (Table I). They showed an absolute requirement of Ca^{2+} for activity, with an optimal concentration of approximately 25 to 50 mM. SDS stimulated their activities about 3-fold, and the maximal stimulation occurred at 0.4 to 0.6 mM SDS. The pH optima for these PLDs were 6.5.

Under the optimal Ca^{2+}, pH, and SDS conditions, PLD 3 showed the lowest affinity toward PC, and PLD 1 had the highest affinity (Table I). The reaction velocities for the PLD

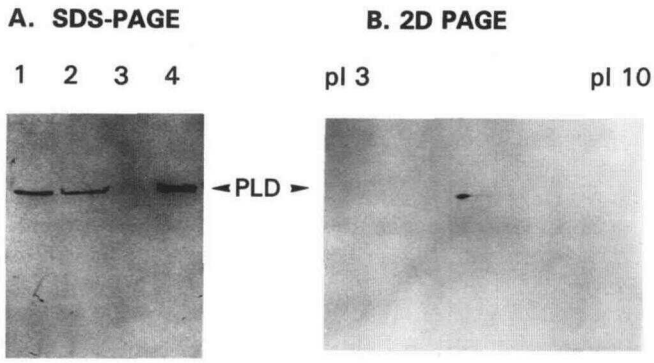

Figure 4. Comparison of PLDs resolved by denaturing PAGE. A, Immunoblot of PLDs resolved on SDS-PAGE. Lanes 1 and 2 contain types 1 and 2 PLDs, respectively, electroeluted from nondenaturing PAGE gels of 2-d endosperm. Lane 3, Protein of 5-d endosperm from the gel slice that corresponded to the position of type 1 PLD in 2-d endosperm. Lane 4, Type 3 PLD from 5-d endosperm. The same amounts of protein (5 μg) were loaded onto each lane. B, Immunoblot of PLDs on two-dimensional (2D) PAGE. The first-dimensional IEF gel contained 9.2 M urea, and the second dimension was 8% SDS-PAGE. Equal amounts of protein from isolated types 1, 2, and 3 PLDs (5 μg each) were mixed and subjected to the two-dimensional PAGE analysis.

Table I. *Comparison of catalytic properties of PLD variants*

PLDs were concentrated by acetone precipitation of 110,000g_{av} supernatant from endosperm of castor bean seeds germinated for 2 and 5 d. PLDs 1, 2, and 3 were separated by nondenaturing PAGE and isolated by electroelution. PLD activities in crude cytosol (110,000g) and partially purified fractions were assayed in the presence of 1% ethanol, and the formation of the combined reaction products PA and PEOH was used to calculate the activities. The specific activities of PLDs 1 and 2 in crude cytosol were estimated based on the relative intensity of densitometric scanning of the PLD 1 and 2 bands on the immunoblot of the nondenaturing PAGE gel (Fig. 1B). PLD 3 in crude cytosol was based on the PLD specific activity in 5-d endosperm. For assessments of pH, Ca^{2+}, and SDS optima and K_m values for PC, all other components in the reaction mixture were the same except for the condition tested. A standard enzyme assay mixture contained 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, 20 μL of PC (0.4 μmol), and 20 μl of enzyme solution in a total volume of 200 μL. Activities were determined by measuring the release of choline from dipalmitoyl-glycero-3-P-[*methyl*-^{3}H]choline. K_m values were estimated by double-reciprocal plots of the activities of PLD variants at various concentrations of PC. Values for K_m are means ± SE of three experiments, and those for the rest are means of two experiments.

PLD Type	PLD Activity		Purification	K_m on PC	Optimal pH	Optimal [Ca^{2+}]	Optimal [SDS]
	110,000g	Purified					
	μmol min^{-1} mg^{-1}		-fold	mM		mM	mM
1	0.018	2.64	147	1.92 ± 0.53	6.5	25–50	0.4–0.6
2	0.022	1.3	59	2.62 ± 0.09	6.0–6.5	25–50	0.4–0.6
3	0.05	6.73	135	5.18 ± 0.26	6.5	25–50	0.6

variants were not assessed because the degree of purity among the variants differed. The PLD variants were partially purified from soluble proteins with the same procedure, and yet the specific activity of PLD 3 on PC was 2- and 5-fold higher than that of PLDs 1 and 2, respectively (Table I; Fig. 5, A and B). This higher specific activity of PLD 3 could result from a higher PLD concentration present in the purified fraction and/or a higher turnover rate of the reaction by PLD 3. Judged from the density of the immunoblot bands of the isolated PLDs, the protein concentration of PLD 3 was higher than that of PLDs 1 and 2 (Fig. 4A), which might account for the high specific activity of PLD 3.

PLDs 1, 2, and 3 exhibited similar substrate specificities; they hydrolyzed PC, PE, and PG (Fig. 5), but their activities on PI and PS were undetectable under the present in vitro assay conditions. However, these variants showed distinct patterns of substrate preferences. The hydrolytic rate of PLD 1 on PE was about 2-fold higher than that of PC and PG (Fig. 5A). The order of activities of PLD 2 on substrates was PE > PG > PC (1.63, 1.0, 0.293 μmol PA min^{-1} mg^{-1} protein, respectively). By contrast, PLD 3 had similar hydrolytic rates on PC, PE, and PG.

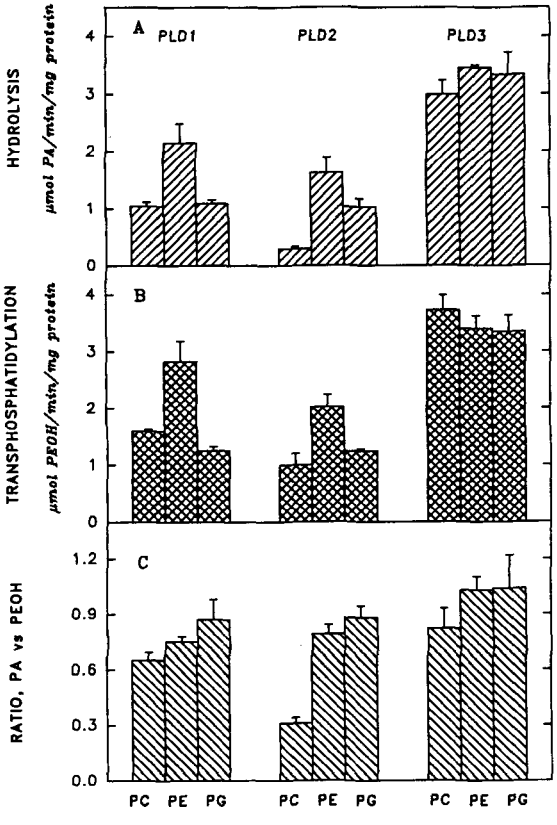

Figure 5. Substrate specificities and preferences of PLD variants. A standard assay mixture contained 1.5 mM phospholipid, 1% ethanol, 100 mM Mes/NaOH (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, 20 μL of substrate (0.4 μmol), and 1 μg of proteins in a total volume of 200 μL. The reaction products PA and PEOH were separated by TLC and quantitated by measuring phosphorus content. A, Formation of the hydrolysis product, PA, by PLD variants; B, formation of the transphosphatidylation product, PEOH, by PLD variants; C, ratio of hydrolysis versus transphosphatidylation from PLD variants. Experiments were repeated three times and individual assays were conducted in duplicate. Values are means ± SE of two experiments.

Transphosphatidylation activity has been proposed as a characteristic of PLD (Heller, 1978), in which primary alcohols substitute for water as the phosphatidate acceptor, and the end lipoidal product of PLD is phosphatidylalcohol. The ratios of hydrolysis versus transphosphatidylation of PLDs 1 and 3 on PC, PE, and PG ranged from 0.7 to 1.0 (Fig. 5), and they were not significantly different from each other. The ratios of PA versus PEOH by PLD 2 on PE and PG were about 0.8. However, the hydrolysis rate for the PLD 2 on PC was 3-fold lower than transphosphatidylation (PA versus PEOH ratio = 0.31) (Fig. 5).

Changes of PLD Variants following Germination and during Leaf Development

PLDs were expressed differentially following seed germination and during leaf development of castor bean. Cytosolic proteins from endosperm of d-0, -1, -2, -3, -4, and -5 germinated seeds were separated on nondenaturing PAGE, followed by both immunoblotting and assaying for PLD activity of the gel slices (Fig. 6). Based on PLD activity assay of the gel slices, PLDs 1 and 2 were present in the endosperm of dry castor bean seeds. A shift from type 1 to 2 PLD in the endosperm was evident after germination was completed. The activity and protein amount of PLD 1 in the endosperm peaked at 2 d and were not detected after 3 d. Both the activity and protein amount of PLD 2 increased in the endosperm during the first 3 d following imbibition. The profiles of the PLD activities were consistent with those of immunoreactive banding patterns. PLDs 1 and 2 in the d-1, -2, and -3 endosperm formed two separate protein bands on an immunoblot of PLD resolved on nondenaturing PAGE. The immunoblot did not reveal distinct PLD bands in the dry seed extract, and this was likely due to the limited amount of PLD protein present in the dry seeds. The type 3 PLD appeared in 4-d endosperm, and the extract showed the same two clustered PLD protein bands as the 5-d endosperm.

During leaf development, PLD protein and activity were highest in young leaves (Fig. 7A). The fact that PLD activity was higher in the young leaves than in the mature ones argues against PLD serving only catabolic functions. There appeared to be a shift of PLD from cytosolic to membrane-associated forms as leaves aged; more than 70% of PLD activity in young leaves was soluble, whereas in fully expanded leaves nearly 80% of the total PLD activity became membrane associated (Fig. 7B). The results of the immunoblot analysis of PLD were in agreement with those of PLD activity measurement. PLD concentration was the highest in cytosolic fractions of young leaves, and the soluble PLD decreased as leaves developed (Fig. 8, A and B). In fully expanded leaves PLD was detected primarily in microsomal fractions. The major PLD forms in the young leaves were types 1 and 2, whereas the mature leaves contained mainly type 2 PLD (Fig. 8C). The type 1 PLD was present largely in the cytosol, whereas type 2 existed in both the soluble and membrane-associated fractions.

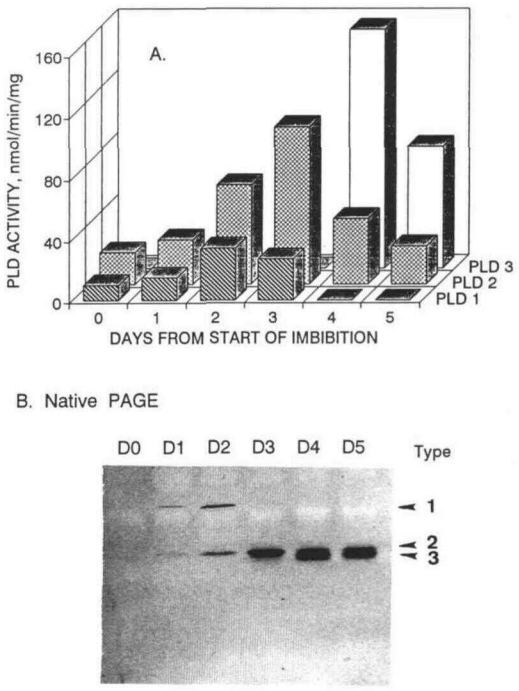

Figure 6. Expression of PLD variants in castor bean endosperm following germination. Cytosolic proteins (110,000g_{av}) from endosperm were acetone precipitated and then separated by nondenaturing PAGE (8%). A, Changes of variant PLD activities. Type 1, 2, and 3 activities were determined from gel slices of a nondenaturing PAGE gel. Type 3 activity in 4- and 5-d endosperm was not distinctly separated from type 2 activity because of the closeness of the type 2 and 3 bands. The amounts of PLD 2 activity in 4- and 5-d endosperm were estimated based on the intensity of the immunoreactive band of PLD 2 relative to that of PLD 3 on nondenaturing PAGE. B, Immunoblot patterns of PLD resolved by nondenaturing PAGE. In both A and B, 50 μg of acetone-precipitated proteins were loaded onto each lane.

DISCUSSION

Structural Heterogeneity of PLD

The present study demonstrates a high degree of structural heterogeneity of plant PLD. The possibility of preparative artifacts seems very unlikely considering the following lines of evidence: (a) The three major forms of PLD in castor bean are resolved independently by nondenaturing PAGE, IEF, and size-exclusion chromatography. (b) These PLD variants are catalytically distinguishable in terms of their substrate affinities and preferences. (c) Expression of these forms follows a well-regulated pattern in different stages of castor bean seedling growth and leaf development. The possibility of more than one PLD has been raised in some of the previous biochemical studies dealing with soluble and membrane-associated PLD activities in both plants and animals. Particulate-associated PLD from cabbage leaves was reported to hydrolyze choline plasmalogen, whereas soluble PLD acted preferentially on ethanolamine plasmalogen (Lands and Hart, 1965; Slotboom et al., 1967). In animals, a membrane-bound PLD acts primarily on PC, whereas a cytosolic form hydrolyzes PC as well as PI, PE, and other phospholipids (Wang et al., 1991). A recent report has shown that a PI-selective PLD in the cytosol is activated when the cell is challenged with an agonist (Huang et al., 1992), and thus it is possible that there is more than one form of this enzyme in the cytosol.

The presence of multiple PLDs at the protein level has not been previously documented in eukaryotes, despite the fact that PLD had been purified from several plant species (Heller et al., 1974; Allgyer and Wells, 1979; Witt et al., 1987; Lee, 1989a). A close look shows that the behavior of PLD during purification in the previous work has actually hinted at the structural heterogeneity of PLD. Two forms of PLD from cottonseeds were observed by both ion-exchange and size-exclusion chromatography (Heller, 1978). During PLD isolation from peanut seeds, SDS-PAGE yielded two sets of proteins of different molecular masses: one in the range of 46 to 50 kD and the other at 98 kD, and the native molecular mass as estimated by size-exclusion chromatography was 200 ± 10 kD. Sedimentation equilibrium ultracentrifugation yielded a minimal molecular mass at 22 ± 3 kD (Heller et al.,

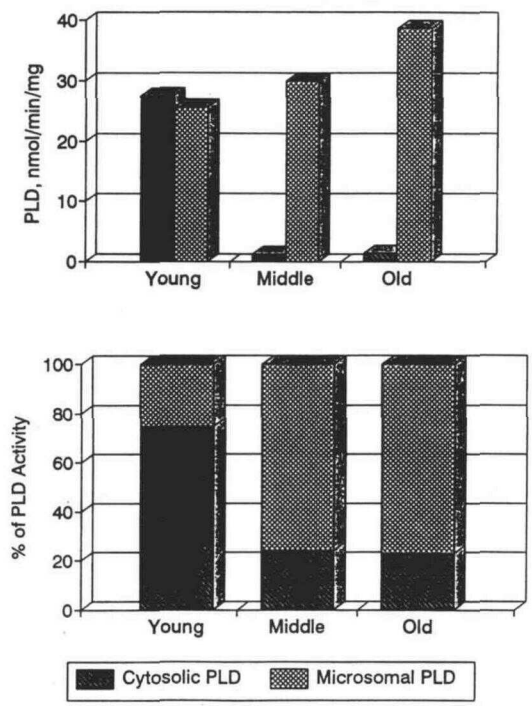

Figure 7. Changes of PLD specific activity (A) and relative distribution of total PLD activity (B) between cytosol and microsomes during leaf development. The cytosolic fraction was the supernatant after centrifugation at 110,000g_{av} for 60 min, and the microsomal fraction was the pellet from the 110,000g_{av} centrifugation of the 10,000g supernatant. Young, Middle, and Old represent three developmental stages of leaves, which are before one-fourth full expansion, full expansion, and 10-d after after full expansion, respectively. The PLD activity was measured using 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-3-glycero-P-choline as substrate in the presence of 1% ethanol. The reaction products PA and PEOH were combined to calculate PLD activity. Experiments were repeated, and the values are means from one representative experiment.

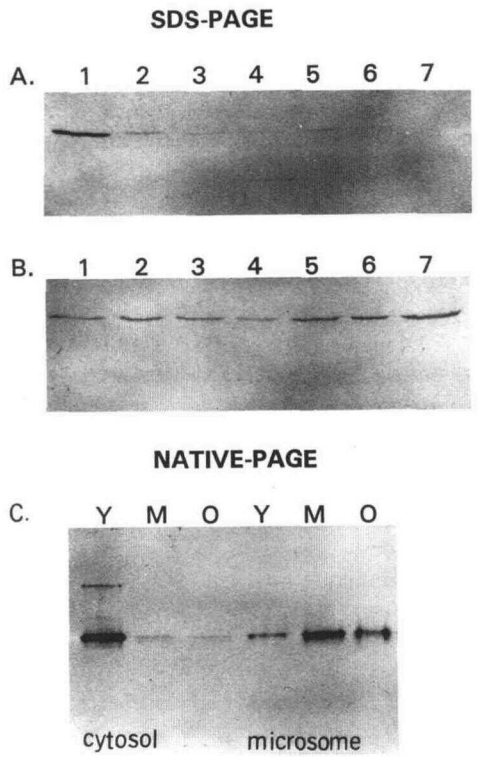

Figure 8. Changes in PLD membrane association and variant pattern during leaf development. A and B, Immunoblots of cytosolic and microsomal PLDs, respectively, resolved by SDS-PAGE (8%). The same amount of protein (50 μg) was loaded in each lane. Lanes 1 to 7 contained protein extracts of leaves in the order from the youngest to the oldest, from one castor bean plant (8 weeks old). C, Immunoblot of PLD variants resolved by nondenaturing PAGE (8%). Y, M, and O were the same leaf material of young, middle, and old as in Figure 7. Y, M, and O on the left are cytosolic proteins, whereas Y, M, O on the right are proteins solubilized from microsomal fractions with 25 mM *n*-octyl β-D-glucopyranoside.

1974). PAGE analysis of the PLD isolated from citrus callus showed two protein bands at about 90 and 94 kD, but it was not established whether both of the proteins were PLD (Witt et al., 1987). Although the bases for these variations were not understood, among those suggested were size transformation, oligomerization, proteolysis, and incomplete purification. The other highly purified PLDs were from cabbage leaves and rice bran, and neither of these PLDs showed apparent structural variations (Allyger and Wells, 1979; Lee, 1989a). This was not surprising because, according to the present study, the expression of PLD variants in the leaves and endosperm of castor bean is growth and developmental stage specific. The type 1 PLD is transitory, appearing in the endosperm of seeds incubated for up to 3 d and in the early developing stage of leaves (i.e. before the leaf is one-fourth fully expanded). The type 3 PLD is present in old tissues, and, furthermore, it is not easily distinguishable from the type 2 PLD if PLD antibodies were not used to detect PLD proteins. The type 2 PLD is often the predominant form of PLD and is present in all growth stages and tissues. If fully expanded leaves had been used as the enzyme source for the purification of castor bean PLD, the type 2 PLD would have been the only form detected.

Origin of PLD Variants

The chemical basis for and molecular composition of the PLD variants are not understood. Multiple molecular forms of any enzyme may originate via genetic or epigenetic processes. The genetic process may begin by gene duplication, followed by mutation to form modified versions of the original enzyme. Multiple forms of an enzyme with epigenetic origins can arise by a number of mechanisms. For example, alternative gene splicing and posttranslational modifications such as phosphorylation, glycosylation, and prenylation are important in regulation of enzyme activity and also lead to the formation of variant forms. Various interactions between polypeptide chains such as disulfide and hydrogen bonding can all modify the conformations of enzymes to give rise to distinct forms. The individual peptide chains may function singly, as parts of large aggregates, or as polymers of various sizes to achieve optimal cellular function. The initial polypeptides can also be shortened by proteolysis to produce different molecules.

The ability to separate the PLDs into multiple forms independently by native PAGE, IEF, and size-exclusion chromatography indicates that they differ in size, pIs, and possibly conformation. On the other hand, immunoblot analyses of one- and two-dimensional SDS-PAGE gels under denaturing conditions using polyclonal antibodies raised against the purified 92-kD protein of PLD detected one protein with the a single molecular mass and pI. Previous studies identified the 92-kD protein as PLD (Wang et al., 1993), but this may not rule out the possibility that some minor components went undetected in previous experiments. The native sizes of the variants as estimated by size-exclusion chromatography are approximately 330, 230, and 270 kD for types 1, 2, and 3, respectively. Thus, the multiple forms observed on nondenaturing PAGE may result from association of multiple components, which might be composed of homo-oligomers (i.e. identical 92-kD subunits) or hetero-oligomers (i.e. association of the 92-kD protein with other components). As a working hypothesis, it is proposed that the same or a similar 92-kD protein is a common catalytic subunit in these variants, and the other components might be involved in activity modulation. Identification of the potential component(s) associated with PLDs requires complete purification of the various molecular forms of PLD.

Growth Stage-Specific Expression of PLDs

The question concerning the functional differences among those molecular forms is of particular interest in relation to the diverse cellular processes possibly involving PLD (Wang, 1993, and refs. therein). These include initiation of lipolytic process during senescence and stress injuries (McCormac et al., 1993; Voisine et al., 1993) and signal transduction (Acharya et al., 1991; Billah, 1993). The present data clearly show that expression of those molecular forms of PLD was well regulated during seed germination and growth and leaf development. Association of the specific PLD variants with

specific physiological stages may shed some light on their possible functional significance. Castor bean endosperm during the first 5 d of imbibition goes from a rapid membrane proliferation stage (first 3 d) to senescence. This uniquely short growth span provides a simple way to capture variant PLD forms. The type 1 PLD may play a role in the rapid growth of plants, since it is expressed in the early stages of seedling establishment and young leaves. A requirement of PLD for seedling growth was proposed for rice (Lee, 1989b). The type 2 PLD appears to be a constitutive form of PLD, since it is found in all of the tissues and stages examined. In the later stages of growth (d 4 and 5) there is a substantial decline in lipid and protein content (Kinney and Moore, 1989). By d 5 after germination, the endosperm seems morphologically and physiologically to be undergoing senescence. Therefore, the type 3 PLD may be related to senescence or stress injuries because it is present in deteriorating endosperm.

Substrate Specificity and Preference of PLDs

Characterization of the catalytic properties of the PLD variants would also help us to understand the role of the structural variation of PLD in the cell. PLDs 1, 2, and 3 hydrolyze PC, PE, and PG but have little activity on PS and PI. The inability of PLD to attack PI in vitro is consistent with previous reports involving in vitro studies of PLD from other plant sources (Heller, 1978). PLD from other plants was shown to be 5- to 10-fold less active on PS than PC or PE (Heller, 1978). However, the present study failed to detect activity of the castor bean PLDs on PS, and the reasons for this are under further investigation.

PLDs 1, 2, and 3 are biochemically distinguishable in terms of their substrate preference and K_m values toward PC. PLDs 1 and 2 hydrolyze PE at a higher rate than PC and PG, whereas PLD 3 has similar rates of activities on PC, PE, and PG. PLDs 1 and 2 are different in their activities on PC and PG; whereas PLD 1 shows no apparent difference in the rate of hydrolysis between PC and PG; the hydrolytic activity of PLD 2 on PC is 3-fold lower than on PG. The differences in substrate preference among the PLD variants may have important functional implications in the cell. For example, activation of PLD 1 and 2 could result in a selective degradation of phospholipids, thereby altering membrane lipid composition. On the other hand, activation of PLD 3, which showed similar rates of activity toward PC, PG, and PE, could lead to general breakdown of membrane phospholipids. This property of PLD 3 would be consistent with the observation that associates PLD 3 with deteriorating and senescent tissues, since the loss of total membrane phospholipids has been reported in those tissues (Voisine et al., 1993).

An important property that remains to be studied is the substrate selectivity of these PLD variants with regard to acyl composition of phospholipids. In this study, the ratios of saturated/monounsaturated/polyunsaturated fatty acids of these phospholipids are: PC and PG, 47.5/30/18.2; PE, 45.3/23.9/23.2; PI, 45.7/6.3/45.5; PS, 44.6/48.9/3.2. The overall ratios of saturated versus unsaturated fatty acids are similar among PC, PE, and PG, and the major difference is probably the higher arachidonate content in PE than PC and PG. The saturated/monounsaturated/polyunsaturated ratios of the two PLD-resistant lipids, PI and PS, are substantially different from those of the PLD-susceptible lipids. PI is high in a polyunsaturated fatty acid, linoleate, and PS is low in polyunsaturated fatty acids but high in a monounsaturated fatty acid, oleate. It is not certain whether a high content of the polyunsaturated fatty acids in PE plays a role in the higher activity of PLDs 1 and 2 on PE than that of PC and PG. It has recently been reported that phospholipases including PLD from castor bean and several other species prefer phospholipids with oxygenated acyl groups (Banas et al., 1992). This substrate preference has been suspected to be involved in protecting membranes from oxidative damage, releasing precursors for hormone synthesis and generating second messengers. It would be of interest to test the substrate specificity and preference toward acyl composition of phospholipids by these PLD forms and to determine which PLD form prefers oxygenated lipids: the growth-associated type 1, the constitutive type 2, or the aging-associated type 3.

It should be noted that, in the experiments characterizing the effects of pH, Ca^{2+}, and PC concentrations on the PLDs, the enzyme activities were assayed by determining the hydrolysis of ^{3}H-labeled dipalmitoyl glycerophosphocholine in the presence of PC from egg yolk. This assay may have influenced the activity measurements if the PLD variants exhibited different degrees of preference for acyl composition of PC.

Elucidation of the catalytic properties of the PLDs in vitro is important in characterizing the structural heterogeneity of PLDs and in providing insights into their cellular roles. However, it should be noted that direct evidence for the substrate specificities and preferences of these PLDs in vivo remains to be obtained. It is unclear whether or not the catalytic properties obtained from the in vitro conditions have direct relevance to the role of these PLD variants in vivo. The in vitro assays used SDS (0.5 mM) and a high concentration of Ca^{2+} (25 mM), and these conditions were obviously quite different from the environment of the PLDs in vivo. It was also reported that the substrate preference of PLD might vary in the absence and presence of activators (e.g. diethyl ether, SDS) in the assay mixtures (Roughan and Slack, 1976). However, the previous studies used a mixture of crude protein extracts or entire leaf tissues. Whether or not the variations were due to selective activation of a given PLD form requires further investigation. On the other hand, these PLD variants might behave similarly in vitro but not be similarly integrated into the molecular ecology of the cell. Their rates of phospholipid degradation and stability within the cell may depend significantly upon their association with other molecules.

In summary, this study provides evidence for the presence of multiple forms of PLD in plants and also demonstrates that the expression of the multiple PLDs is associated with different growth stages. Further investigations of the molecular origin of these forms and role of the structural heterogeneity of PLD in cellular metabolism should advance the current understanding of the regulatory mechanisms and physiological significance of the major lipolytic enzyme in plants.

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