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Structural Heterogeneity of Phospholipase D in 10 Dicots

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The occurrence of multiple forms of phospholipase D (EC 3.1.4.4) was investigated in different tissues of castor bean (*Ricinus communis*) and in other plant species. Phospholipase D variants were resolved by nondenaturing and isoelectric focusing polyacrylamide gel electrophoresis and detected by immunoblotting using anti-phospholipase D antibodies and by enzyme activity assay. Three phospholipase D variants were produced differentially in the roots, endosperm, cotyledons, and hypocotyl of 5-day postgermination seedlings of castor bean. Furthermore, different patterns of phospholipase D variants were found in the different regions of hypocotyl (elongated and hook). Multiple phospholipase D forms were found in florets of cauliflower and broccoli, leaves of cabbage, celery, tomato, and potato, and alfalfa sprouts, suggesting that structural heterogeneity of phospholipase D occurs widely in plants. © 1996 Academic Press, Inc.

Phospholipase D (PLD; EC 3.1.4.4), which hydrolyzes phospholipids to generate phosphatidic acid and a free alcohol group, has been suggested to play an important role in a number of cellular processes. In animals, many receptor agonists activate PLD-mediated breakdown of phospholipids, particularly phosphatidylcholine (PC) [1]. Such activation of PLD is thought to be a regulatory step in cell proliferation, exocytosis, respiratory burst, membrane trafficking, and secretion [1, 2]. Several mechanisms including protein kinase C, G proteins, Ca^{2+} flux, and receptor-linked tyrosine kinase have been proposed for activation in response to different agonist treatments [2]. In plants the involvement of PLD has been implicated in the physiological processes such as membrane deterioration during senescence, aging, and stress injuries and lipid mobilization during seed germination [3, 4, 5, 6, 7].

The regulation and physiological role of PLD are, however, yet to be fully understood. A recent study demonstrated the presence of multiple forms of PLD in castor bean [8]. Three major PLD variants in *Ricinus* tissues can be separated from each other by nondenaturing polyacrylamide gel electrophoresis, isoelectric focusing, and size exclusion chromatography. These variants were also distinguishable in terms of their affinity for PC and selectivity for different classes of phospholipids. The appearance of specific PLD variants was associated with different growth stages of endosperm and leaf development, suggesting that they may play different roles in mediating various cellular functions [8, 9]. The objectives of the present study were to establish whether various PLD forms are expressed differentially in different tissues of castor bean and whether the molecular heterogeneity also occurs in other plant species.

EXPERIMENTAL

Biologicals and chemicals. Coatless seeds of castor bean were germinated at 30°C in the dark for 5 days [10]. Roots, cotyledon, hypocotyl, and endosperm were harvested for protein extraction. Cauliflower, broccoli, cabbage, celery, soybean and alfalfa sprouts were obtained from a local grocery store. Tomato, potato, and tobacco plants were grown under cool fluorescent lights at 23 ± 3°C. Polyclonal PLD antibodies were raised in rabbits against a purified 92 kDa protein with PLD activity from 2-d postgermination endosperm of castor bean [10]. Radioisotopes, chemicals, and other reagents were obtained from the sources previously reported [10].

Protein extraction and separation of PLD variants. Different tissues (ca. 1 g each) from 5-d postgermination seedlings of castor bean were harvested separately and ground in liquid nitrogen with a mortar and pestle into fine powder. Proteins were extracted by stirring the powder in a buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM KCl, 1 mM EDTA, 0.5 mM

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PMSF, and 2 mM DTT on ice. The homogenate was centrifuged at 20,000 × g at 4°C for 20 min. The supernatant was transferred to a fresh tube and used for PLD variant separation. The procedure for extracting proteins from other plant species was the same as that above except that polyvinylpolypyrollidone (10%, w/v) and 10 mM DTT were added to the extraction buffer. This modification improved the resolution of PLD variants in these plants. Protein content was determined with a dye-binding assay according to manufacturer's instructions (Bio-Rad).

PLD variants were resolved on nondenaturing polyacrylamide gels and IEF gels as previously described [8]. Immunoblot analysis and PLD activity assay were performed as previously described [8].

RESULTS AND DISCUSSION

PLD Variants in Different Tissues of Castor Bean Seedlings

Three types of PLD were previously identified and partially characterized in castor bean endosperm and leaves [8]. They were designated as PLDs 1, 2, and 3 according to their order of mobility during nondenaturing polyacrylamide gel electrophoresis (PAGE). To examine whether the patterns of PLD variants occur differentially in various tissues of castor bean, the present study analyzed proteins from cotyledons, endosperm, roots, elongated and hook segments of hypocotyl of 5-d postgermination castor bean seedlings. PLDs were separated by nondenaturing PAGE, followed by immunoblotting using PLD antibodies (Fig. 1A). PLDs 2 and 3 were resolved into two clustered immunoreactive bands in endosperm, which was consistent with the previous observations [6]. Cotyledons exhibited three PLD variants with the PLD 1 being the major one, whereas only PLD 2 was detected in roots. Different regions of hypocotyl showed different PLD variants; the elongated region contained only PLD 2 whereas the hook region had both PLDs 1 and 2.

The immunoreactive bands were previously documented to have PLD activity [8]. The antibodies have been shown to be PLD-specific and are able to precipitate a 92 kDa PLD protein from crude protein extract of castor bean [10]. The 92 kDa protein has been unequivocally demonstrated to be a catalytically active PLD because cDNA of this protein has been cloned and expression of

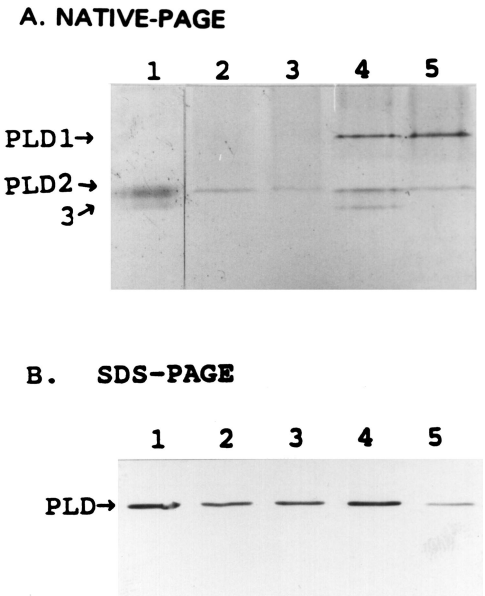

FIG. 1. Immunoblotting analysis of PLD resolved on nondenaturing PAGE (A) and SDS–PAGE (B) from different organs of 5-d postgermination seedlings of castor bean. Lane 1, endosperm; lane 2, roots; lane 3, elongated segment of hypocotyl; lane 4, cotyledons; lane 5, hypocotyl hook. Proteins of 20,000 × g supernatant (40 μg/lane) were loaded onto 8% nondenaturing PAGE. For SDS–PAGE 30 μg protein was loaded in lanes 1, 2, and 3, and about 20 and 10 μg protein was in lanes 4, and 5, respectively. The blots were made visible using alkaline phosphatase conjugated to goat antibodies against rabbit immunoglobulin.

the cDNA in *E. coli* yielded an active PLD [11]. However, the immunoblotting could not distinguish PLD variants under denatured condition. After proteins from the different tissues were subjected to SDS-PAGE, one 92 kDa band was visible on the immunoblot (Fig. 1B).

The ability to separate the PLDs into multiple forms by nondenaturing PAGE indicates that they may differ in size, isoelectric points, and/or conformation. The inability to separate PLD variants on SDS-PAGE suggests that the same or similar 92 kDa protein is a common catalytic subunit in these variants, and that the different forms do not result from proteolytic cleavage of the 92 kDa protein. The different forms may not come from glycosylation and lipid attachments since the primary sequence of PLD lacks the consensus sequences for such modifications [11]. A previous study showed that the native sizes of the three PLD variants, as estimated by size-exclusion chromatography, were approximately 330, 230, and 270 kDa for PLDs 1, 2, and 3, respectively [8]. 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 kDa subunits) or hetero-oligomers (i.e. association of the 92 kDa subunit with other components). It is possible that the other factors may associate with the 92 kDa catalytic protein and modulate PLD activity.

The association of specific PLD variants with different growth stages [8] has led to the hypothesis that PLD 1 is involved in membrane proliferation during rapid plant growth, PLD 2 is

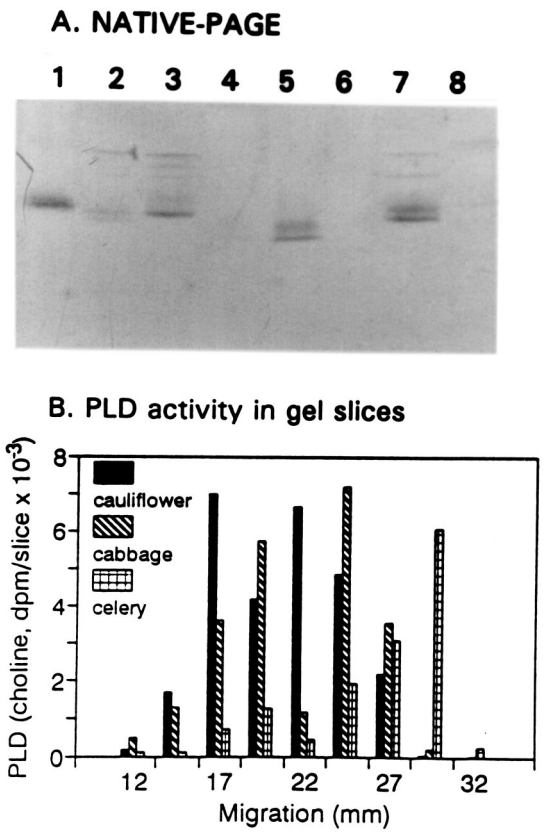

FIG. 2. Nondenaturing PAGE separation of PLDs from different plants. A. Immunoblotting analysis with PLD antibodies: lane 1, endosperm of 5-d postgermination castor bean; lane 2, broccoli florets; lane 3, cauliflower florets; lane 4, old celery leaf; lane 5, young celery leaf; lane 6, old cabbage leaf; lane 7, young cabbage leaf (see text); lane 8, endosperm of 2-d postgermination castor bean. The same amounts of proteins (65 μg/lane) were loaded onto 8% PAGE. The blots were made visible using alkaline phosphatase conjugated to goat antibodies against rabbit immunoglobulin. B. PLD activity profile assayed in gel slices (2.5 mm/slice).

constitutive and plays a role in lipid turnover for maintaining normal growth and development, while PLD 3 takes part in lipid degradation during membrane deterioration and senescence. The present results found that PLD 2 was present in all the organs examined whereas PLDs 1 and 3 were associated with specific tissues. The occurrence of PLD 1 in hook, but not in the elongated region of hypocotyl supports the notion that PLD 1 is associated with rapid growth. Cells in the hook region are metabolically much more active than the mature cells in the elongated region, and the hook in a seedling often is the region most responsive to hormonal and environmental changes. The presence of all three PLD variants in cotyledons may reflect a complex metabolism in this tissue. However, the putative PLD 3 band in the cotyledon migrated slightly faster that of PLD 3 characterized in endosperm. It is unclear whether it represents a new PLD variant in cotyledon.

PLD Variants in Other Plant Species

To determine whether the structural heterogeneity of PLD also occurs in other plants, PLD banding patterns were investigated in florets of cauliflower and broccoli, and young and old leaves of green cabbage and celery. Young leaves refer to newly emerging leaves from the heart of the cabbage or celery, while old leaves are tissue from the outermost layer of the cabbage or expanded leaves from the ends of fully grown celery stalks. Immunoblotting analysis of proteins resolved on nondenaturing-PAGE using anti-PLD antibodies revealed multiple bands in the soluble proteins of these plants (Fig. 2A). Broccoli and cauliflower florets and young cabbage leaves each showed several immunoreactive bands, whereas young celery leaves had only two bands. No discrete PLD bands were detected in the old leaves of cabbage and celery because of the low amount of PLD protein in these tissues (see below). Each species exhibited a different pattern of PLD variants, and yet two subspecies of *Brassica oleracea*, broccoli and cauliflower, shared 3 bands with the same mobility on the nondenaturing PAGE. The other *B. oleracea* subspecies, cabbage, also showed a pattern of multiple PLD variants similar to that of broccoli and cauliflower, whereas castor bean and celery showed much different PLD variant patterns from the *Brassica* species. The observation that taxonomically related plants share a more similar variant pattern than with distant species further support the validity of the methodology for separating the PLD variants.

To confirm those immunoreactive bands as PLD, a parallel gel was run and sliced (2.5 mm/slice) for measurement of PLD activity (Fig. 2B). The match of the positions of the gel slices that exhibited PLD activity with those of the immunoreactive bands indicates that those immunopositive bands result from the presence of PLD protein. The minor PLD activity in the gel regions where immunoreactive PLD bands were invisible could be caused by residual vertical streaking effect of proteins in the gel, which was noticeable when an immunoblot of the gel was overdeveloped.

The multiplicity of PLD was further analyzed by isoelectric focusing (IEF) gel electrophoresis

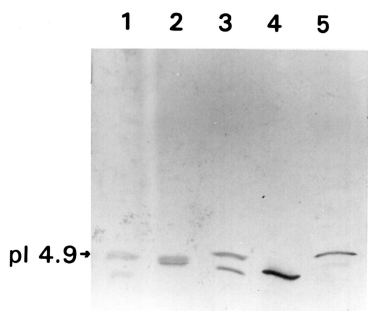

FIG. 3. Immunoblotting analysis of nondenaturing isoelectric focusing separation of PLDs from different plants. Proteins (65 μg/lane) were separated on pH ranging from 3 to 10 (top to bottom). Lane 1, endosperm of 5-d postgermination castor bean; lane 2, broccoli florets; lane 3, cauliflower florets; lane 4, young celery leaf; lane 5, young cabbage leaf.

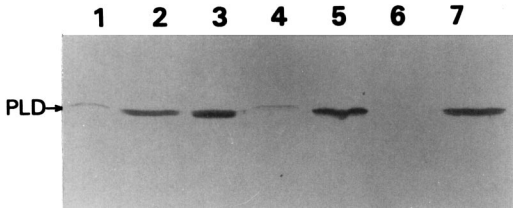

FIG. 4. Immunoblotting analysis of PLD resolved on SDS–PAGE from different plants. Lane 1, endosperm of 5-d postgermination castor bean; lane 2, broccoli florets; lane 3, cauliflower florets; lane 4, old celery leaf; lane 5, young celery leaf; lane 6, old cabbage leaf; lane 7, young cabbage leaf (see text). The same amounts of proteins (65 μg/lane) were loaded on 8% SDS–PAGE except castor bean endosperm for which 20 μg protein was used.

under nondenaturing conditions. PLD from 5-d postgermination castor bean endosperm, broccoli florets, cauliflower florets, young celery leaves, and young cabbage leaves all exhibited acidic pIs (Fig. 3). PLD from 5-d postgermination endosperm gave two bands at pIs around 4.7 and 4.9. Two clustered PLD bands at pI of 4.9 were observed in broccoli, two distinct PLD bands at pIs of 4.9 and 4.8 were in cauliflower, one PLD band at pI of 4.8 was in celery, and two (one strong and one weak) bands at pIs of 5.0 and 4.9 were in cabbage. The presence of PLD in those immunoreactive bands was confirmed by PLD activity occurring in the corresponding gel slices of a parallel IEF gel (data not shown).

The IEF separation revealed fewer PLD variants than that of nondenaturing PAGE, and the number of immunoreactive bands detected by the latter method was twice as many as the IEF in broccoli, cauliflower, celery, and cabbage. Such a difference in the number of PLD variants resolved between the two methods may result from their principles of separation because IEF separated PLD based on their isoelectric points, whereas nondenaturing PAGE resolved PLDs based on their size, isoelectric points, and conformation.

When proteins from these species were subjected to SDS-PAGE, only one immunoreactive band at around 90–92 kDa was visible (Fig. 4). This observation was the same as that in castor bean, indicating that PLD variants contain the same or very similar 92 kDa proteins as discussed above. In addition, these results showed that the multiple forms resolved under nondenaturing conditions did not result from proteolytic cleavage of the 92 kDa PLD protein.

The survey on PLD variant patterns was expanded into other plant species including tomato, potato, tobacco, soybean, and alfalfa. Immunoblotting of nondenaturing PAGE showed 2 PLD bands in tomato leaves, 3 in potato leaves, 1 in tobacco leaves, 2 in alfalfa sprouts, and 1 in soybean sprouts (Fig. 5). As already shown in castor bean, PLD variants were expressed differentially in various tissues (Fig. 2A) and during development [8]. Therefore, this survey using one or two tissues of each plant species by no means revealed the total number of PLD variants in the different species examined. In addition, this result showed that young leaves of tomato, potato, and tobacco (newly emerging leaves at top of the plants) all contained higher amounts of soluble PLD than did

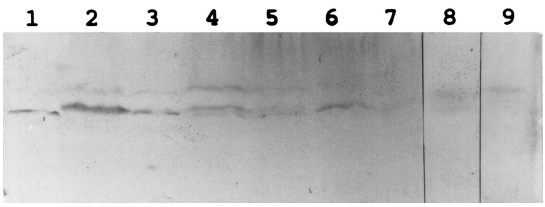

FIG. 5. Immunoblotting analysis of PLD variants resolved on nondenaturing PAGE from different plant species. Lane 1, castor bean leaf; lanes 2 and 3, young and old tomato leaves; lanes 4 and 5, young and old potato leaves; lanes 6 and 7, young and old tobacco leaves (see text); lane 8, alfalfa sprouts; lane 9, soybean sprouts. The same amounts of proteins (65 μg/lane) were used.

older leaves (fully expanded leaves at bottom of the plants), which was consistent with the observations on castor bean [8], cabbage, and celery (Figs. 2A, 4).

In summary, the present study demonstrates that structural heterogeneity of PLD occurs widely in plants and that the presence of PLD variants is tissue-specific and follows a well-regulated pattern in different stages of growth and development. Further studies are needed to understand the molecular origin and the physiological roles of the PLD variants in plants.

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