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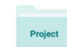

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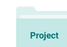

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Chapter 19

Assaying Different Types of Plant Phospholipase D Activities In Vitro

Kirk L. Pappan and Xuemin Wang

Abstract

Over the past decade, tremendous progress has been made toward understanding the physiological functions of individual members of the diverse phospholipase D (PLD) family of enzymes in plants. For instance, the involvement of plant PLD members has been shown or suggested in a wide variety of the cellular and physiological processes such as regulating stomatal opening and closure; signaling plant responses to drought, salt, and other abiotic and biotic stresses; organizing microtubule and actin cytoskeletal structures; promoting pollen tube growth; cycling phosphorus; signaling nitrogen availability; regulating *N*-acylethanolamine stress signaling; and remodeling membrane phospholipids in plant responses to phosphate deprivation and during and after freezing. There are at least a dozen PLDs in *Arabidopsis* that can be separated into six classes, phospholipases Dα, Dβ, Dγ, Dδ, Dε, and Dζ, based on their molecular and enzymatic characteristics. Several of the classes have distinguishing enzymatic properties that can be used to discriminate among the various classes. Here we provide four variations of in vitro PLD activity assays using choline-labeled phosphatidylcholine to distinguish, to the extent possible, among the different PLD classes.

Key words *Arabidopsis*, Plant, Phospholipase D, PLD, Classes, Isoforms, In vitro assay, Activity assay, Protocol, Method

1 Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of glycerophospholipids into phosphatidic acid (PA) and a free headgroup. For instance, PLD's hydrolysis of phosphatidylcholine (PC) generates PA and choline. There are currently 12 PLD genes known in *Arabidopsis* (1) and similar or greater diversity exists in many plants including grape and poplar (2), rice (3), and poppy (4). The complexity of the PLD family has necessitated the use of functional genomics and gene silencing techniques, in addition to biochemical assay methods, to elucidate individual PLD functions. Even with genetic techniques and the availability of isoform-specific antibodies, measurement of PLD activity remains a major approach for detecting and identifying PLD in plants. Within certain limitations,

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the distinct cofactor and activating conditions of different classes of PLDs offer a way to distinguish PLD classes from one another. The goal of this report is to describe key features of the major PLD classes, based on our current understanding of *Arabidopsis*, and to describe assay methods pertinent to these classes.

Phylogenic analysis places the 12 *Arabidopsis* PLDs into six classes of variable size (1), and, for the most part, these classes differ from each other by catalytic properties (see Table 1). For instance, classes vary by their dependence on polyphosphoinositides (PPI), narrowly defined here as phosphatidylinositol-4, 5-bisphosphate (PIP_2) or phosphoinositol-4-phosphate (PIP), calcium, oleic acid, and phospholipid substrates. The properties of the *Arabidopsis* PLD classes that are particularly relevant to measuring their activity by in vitro activity assay are briefly reviewed below.

PLDα: PLDα family members are involved in maintaining proper water balance, including responding to drought (5), salt (6), and freezing stresses (7), and in responding to pathogens (8, 9). The three members of PLDα comprise the iconic class of PLD that is active at 20–100 mM Ca^{2+} without detergent or down to 5 mM Ca^{2+} with detergents. At millimolar calcium, PLDα members hydrolyze PC, phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) without requiring mixed phospholipid vesicles or PPI. In addition to these iconic activating conditions, PLDα members have recognized activity at more physiologically relevant conditions (10). At a moderately acidic pH, PLDα can hydrolyze PC in the presence of mixed lipid vesicles containing PE, PPI, and calcium as low as 50 μM. PLDα members have calcium-binding C2 domains but lack the full complement of acidic amino acid residues believed to coordinate Ca^{2+} binding.

PLDβ and PLDγ: Little is known about the functions of PLDβ and PLDγ family members but both classes have been implicated in early responses to pathogens and PLDγ may have a role in signaling leading to hypersensitive responses (9). These two PLD classes may also be involved in regulating PLDα since both efficiently utilize *N*-acylphosphatidylethanolamine (NAPE), generating phosphatidic acid and *N*-acylethanolamine (11), the latter of which is a potent PLDα inhibitor (12). Real-time PCR profiling indicated that *PLDγ1* is predominately expressed in roots whereas *PLDγ2* and *γ3* expression was strong in inflorescence stems but nearly absent in roots (13). Members of these two PLD classes were the first non-PLDα-class enzymes described in plants and are characterized by activity toward a broad range of phospholipid substrates in the presence of PPI, PE, and micromolar calcium (14, 15). The C2 calcium-binding domain and Lys/Arg-rich PIP_2-binding motifs of PLDβ and PLDγ members are prototypical in structure and may explain their activity at low calcium concentrations and PPI-dependence (16). In practice, it is not possible to design in vitro assays that discriminate PLDβ from PLDγ, but it is possible to distinguish them from other classes.

Table 1
Signature enzymatic properties and molecular features of *Arabidopsis* PLD classes

Arabidopsis PLD classes (members)	Known cofactor(s)	Known substrate(s)	Distinguishing molecular features	References
PLDα (1,2,3)	Ca^{2+} (mM) and detergent (pH 6.5) or Ca^{2+} (μM) and PIP_2 (pH 5.0)	PC, PE, PG	C2 domain with fewer acidic residues; one K/R-rich motif	(10, 11, 16)
PLDβ (1,2)	Ca^{2+} (μM), PIP_2 or PIP, and PE	PC, PE, PG, NAPE > PS	C2 domain; two K/R-rich motifs	(11, 15)
PLDγ (1,2,3)	Ca^{2+} (μM), PIP_2 or PIP, and PE	PE, NAPE, PG > PC, PS	C2 domain; two K/R-rich motifs	(11, 16)
PLDδ	Oleic acid, Ca^{2+}	PE > PC	One K/R-rich motif; R-399; C2 domain	(22, 23)
PLDε	Cofactors similar to those of PLDα = PLDδ > PLDβ	PC > PE > PG	Altered C2 domain lacking acidic residues; partial K/R-rich motif	(1, 24)
PLDζ (1,2)	PIP_2	PC	PH and PX domains; K/R-rich motif	(1)

*PLD*δ: PLDδ increases freezing tolerance (7, 17, 18), regulates cytoskeletal organization (19), helps plants cope with drought stress (20), and improves plant stress tolerance by dampening H_2O_2-induced apoptosis (21). This enzyme is optimally active in the presence of its substrate, PC, mixed with 0.5 mM oleic acid and 100 μM Ca^{2+}, whereas PLDα, PLDβ, and PLDγ1 are inactive under these conditions (22). Polyunsaturated fatty acids, linoleic acid and linolenic acid, as well as PIP_2 stimulate PLDδ about half as well as monounsaturated oleic acid (22) and the enzyme prefers PE to PC as a substrate (23).

*PLD*ε: Prior to its biochemical characterization, PLDε was tentatively designated as PLDα4 based on its shared gene architecture with PLDα1–3 (1) but has recently been reclassified as PLDε due to its unique biochemical and sequence properties (24). Of all the PLD classes in *Arabidopsis*, PLDε has the most permissive activation conditions, showing activity under conditions appropriate for detecting PLDα (50 mM Ca^{2+}, SDS, and PC), PLDδ (100 μM Ca^{2+}, 0.6 mM oleate, and PC), and PLDβ/γ (PE/PIP_2/PC and 50 μM Ca^{2+}). However, PLDε has a clear calcium requirement and shows no PC hydrolysis activity using calcium-free PLDζ assay conditions (PC and PIP_2; no calcium). The broad calcium-dependent activity of this enzyme could stem from alterations in both its C2 calcium-binding domain and Lys/Arg-rich motif (1). Over-expression of PLDε leads to increased nitrate uptake, biomass accumulation, and root elongation whereas knockouts of PLDε display stunted growth and lessened root elongation (24).

*PLD*ζ: PLDζ is distinct among the PLD enzymes in *Arabidopsis* by virtue of its calcium-independent activity and the presence of Phox homology (PH) and pleckstrin homology (PX) domains in lieu of the calcium-binding C2 domain found in the other plant PLD classes (1). The PH and PX domains are common to mammalian PLDs but are absent in the other *Arabidopsis* PLD classes. Some PX domains can bind PIP_2 and SH3 adaptor proteins and PH domains appear to bind PPI. Although a role in membrane targeting can be speculated, the significance of these domains in PLDζ remains to be demonstrated. PLDζ requires PIP_2, but not calcium, for activity. In fact, 1 mM Ca^{2+} inhibits nearly 75 % of PLDζ activity and 10 mM Ca^{2+} completely inhibits it, which offers an interesting contrast to PLDα's preference for millimolar calcium. PLDζs are involved in conserving and recycling phosphorus under phosphorus-limited growth conditions by initiating the remodeling pathway that converts phospholipids into galactolipids (25, 26). They also play a role in vesicular trafficking and auxin response (27).

2 Materials

2.1 Lipids and Other Materials

PC (Sigma-Aldrich, Cat. No. P3556; average MW 768 g/mol), PE (Avanti Polar Lipids, Cat. No. 840021; average MW 747 g/mol), and PIP_2 (Avanti Polar Lipids, Cat. No. 840046; average MW 1,098 g/mol) can be obtained from the indicated sources. Dipalmitoylglycero-3-*P*-[*methyl*-^{3}H]choline is available from Perkin Elmer. Other chemicals are available from vendors such as Fisher Scientific or Sigma-Aldrich. All solutions should be prepared with laboratory-grade deionized water except for lipid stock solutions that are prepared in solvents as indicated. Tritium should be used in a dedicated radioactivity area and following each use, benches, pipettes, centrifuges, and other equipment should be cleaned with alcohol or Count Off decontamination solution and the area and equipment monitored for radioactivity by swab testing.

2.2 Lipid Stock Solutions

1. Phosphatidylcholine stock solution: 100 mg/ml and 10 mg/ml 1,2-diacyl-*sn*-glycero-3-phosphocholine (i.e., egg yolk PC) in chloroform (*see* **Note 1**).
2. Phosphatidylethanolamine stock solution: 25 mg/ml L-α-phosphatidylethanolamine (i.e., egg PE) in chloroform to a stock (*see* **Note 2**).
3. PIP_2 stock solution: 5 mg/ml L-α-phosphatidylinositol 4, 5-bisphosphate (i.e., porcine brain) in 20:9:1 chloroform: methanol:water (*see* **Note 3**).
4. Oleate stock solution: 15 mg/ml oleic acid in chloroform.

2.3 Assay Reaction Buffers and Other Components

1. PLDα reaction buffer (A buffer; 10×; see Subheading 3.1 below): 1 M 4-morpholineethanesulfonic acid (MES; pH 6.5) and 0.25 M $CaCl_2$.
2. PLDβ/γ/δ reaction buffer (BGD buffer; 10×; see Subheadings 3.2 and 3.3 below): 1 M Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl, pH 7.0), 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.8 M KCl.
3. PLDζ reaction buffer (Z buffer; 10×; see Subheading 3.4 below): 1 M Tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl, pH 7.0) and 0.8 M KCl.
4. 5 mM sodium dodecyl sulfate (SDS).

2.4 Phospholipid Substrate Emulsions

1. PLDα substrate (10×; see Subheading 3.1 below)—For 1 ml of substrate (enough for ~100 reactions), mix 150 μl of 100 mg/ml egg yolk PC with 2 μl of 1 μCi/μl dipalmitoylglycero-3-*P*-[*methyl*-^{3}H]choline in a 1.5 ml microcentrifuge tube (*see* **Note 4**). An aliquot of this 20 mM stock PC substrate will be used to obtain a final working concentration of 2 mM PC in PLD reactions.

2. PLDβ/γ substrate (10×; *see* Subheading 3.2 below)—For 1 ml of substrate (enough for ~100 reactions), mix 104 μl of 25 mg/ml PE, 68 μl 5 mg/ml PIP_2, 16.5 μl 10 mg/ml PC, and 2 μl of 1 μCi/μl dipalmitoylglycero-3-*P*-[*methyl*-^{3}H]choline in a 1.5 ml microcentrifuge tube (*see* **Note 4**). The total phospholipid concentration of this stock PE (87.5 mol %), PIP_2 (7.5 mol %), and PC (5 mol %) substrate is 4 mM and a final concentration of 0.4 mM is used in PLD reactions.
3. PLDδ substrate (10×; *see* Subheading 3.3 below)—For 1 ml of substrate (enough for ~100 reactions), mix 115 μl of 10 mg/ml egg yolk PC, 113 μl 15 mg/ml oleic acid, and 2 μl of 1 μCi/μl dipalmitoylglycero-3-*P*-[*methyl*-^{3}H]choline in a 1.5 ml microcentrifuge tube (*see* **Note 4**). An aliquot of this stock 1.5 mM PC and 6 mM oleate substrate will be used to obtain a final working concentration of 0.15 mM PC and 0.6 mM oleate in PLD reactions.
4. PLDζ substrate (10×; *see* Subheading 3.4 below)—For 1 ml of substrate (enough for ~100 reactions), mix 57 μl 5 mg/ml PIP_2, 29 μl 100 mg/ml PC, and 2 μl of 1 μCi/μl dipalmitoylglycero-3-*P*-[*methyl*-^{3}H]choline in a 1.5 ml microcentrifuge tube (*see* **Note 4**). The total phospholipid concentration of this stock (93.5 mol %) PC and (6.5 mol %) PIP_2 is 4 mM and a final concentration of 0.4 mM is used in PLD reactions.

2.5 PLD Protein Source

Plant PLD can be prepared from plant tissues or recombinant protein expressed in *Escherichia coli* in varying degrees of purity ranging from crude preparations to highly purified forms. It is important to note that different PLDs are associated with different subcellular fractions (22, 28). For example, PLDα is associated with both soluble and membrane-particulate fractions and its allocation between soluble and membrane-associated fractions changes during leaf development and in response to stress, such as wounding (29, 30). PLDβ, γ, δ, ε, and ζ are all primarily associated with membrane, but differ some in respect to specific membranes and the strengths of their membrane associations. PLDγs are associated mostly with intracellular membranes, PLDε is predominantly associated with the plasma membrane (14, 16), whereas PLDδ is exclusively associated with the plasma membrane (22). PLDζ2 is found in tonoplasts whereas PLDζ1 is likely to be associated with the plasma membrane. A major portion of PLDα, β, and γ can be dislodged from membrane fractions by salt treatment of the membranes (22). On the other hand, solubilization of PLDδ requires detergent. Readers are referred to other reports for detailed information on purification or fractionation of PLDα (27), PLDδ (22), and PLDε (24) as well as recombinant expression and purification of PLDα, PLDβ, and PLDγ (13, 16); PLDδ (22); and PLDζ (1).

The main focus of this chapter is to describe assays capable of discriminating among the classes of plant PLD, so given below is a

brief general protein extraction procedure for preparing crude-soluble and peripheral membrane PLD fractions (14). It is useful to note also that *E. coli* contain no detectable PLD activities. Given the lack of background PLD activity, the activities of various recombinant PLDs expressed in *E. coli* can be detected directly using cell lysates without any purification.

1. Grind leaf or other plant tissue in a chilled mortar and pestle at a ratio of one part tissue (mg) to three parts of a homogenization buffer (ml) containing 50 mM Tris–HCl (pH 7.5), 10 mM KCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM dithiothreitol at 4 °C.
2. Centrifuge the homogenate at 2,000 × *g* for 10 min at 4 °C to remove cellular debris. Keep supernatants.
3. Centrifuge the supernatant at 100,000 × *g* for 45 min at 4 °C. Remove the supernatant containing soluble proteins. The pellet contains microsomal proteins that can be further fractionated by salt extraction.
4. Resuspend the pellet in salt-extraction buffer consisting of 0.44 M KCl in the homogenization buffer. Incubate for 1 h at 4 °C and then collect the salt-insoluble materials by centrifugation at 100,000 × *g* for 45 min.
5. Remove the supernatant containing the peripherally membrane-associated proteins.
6. Measure the protein content in the soluble and membrane-associated protein fractions by the Bradford or other suitable protein quantification method.
7. Activity assays can be performed using 1–20 μg per reaction. Fractions can be used directly or stored in aliquots at −80 °C.

2.6 Reaction Stop and Extraction Solutions

1. 2:1 (v/v) chloroform:methanol.
2. 2 M KCl.

3 Methods

Described below are four basic in vitro assay methods that can be used to measure the known classes of PLD in *Arabidopsis*. Each assay has been given a descriptive name and lists, to the extent possible, which classes are active and/or inactive under the specific assay conditions. The four methods use PC with a radiolabeled choline headgroup for relatively rapid and moderately high-throughput screening.

3.1 Iconic High Ca^{2+}-Dependent PLD Assay

This assay can be used to detect PLDα and PLDε enzyme classes. Other classes have no detectable activity under these conditions (i.e., millimolar calcium).

1. A single reaction contains the following components.

1× (μl)	Reaction component
10	10× A buffer
10	10× PLDα substrate
10	5 mM SDS
X	PLD protein source
Y	H_2O
100	Total reaction volume

2. Determine the number of reactions (factoring in replicates and including blanks and controls) and prepare a master mix, omitting the protein source, in slight excess of that number (*see* **Note 5**).
3. Transfer the appropriate volume of master mix, to individual 1.5 ml microcentrifuge reaction tubes, such that the final reaction volume will be 100 μl when the protein sample is added (*see* **Note 6**).
4. Initiate the reaction by adding the PLD protein source, mixing with a brief vortex pulse, and incubate in a shaking water bath for 30 min at 30 °C.
5. Stop the reaction by adding 1 ml of 2:1 (v/v) chloroform:methanol and 100 μl of 2 M KCl.
6. Cap tubes tightly, vortex for ~10 s, and centrifuge at 12,000 × *g* for 5 min.
7. Remove a 200 μl aliquot of the aqueous upper phase, mix with 3 ml of scintillation fluid, firmly cap the scintillation vial, vortex vigorously, and measure the release of [^{3}H]choline by liquid scintillation counting.

3.2 PIP_2- and Ca^{2+}-Dependent PLD Assay

PLDβ, PLDγ, and PLDζ classes exhibit robust activity under the described reaction conditions below, but if Ca^{2+} is omitted, only PLDζ will retain its activity. PLDα has minimal activity under these conditions (i.e., pH 7) but has considerable activity against this mixed phospholipid substrate at pH 5. PLDδ and PLDε have limited activity representing about 25 % of their maximal activity under their preferred assay conditions.

1. A single reaction contains the following components.

1× (μl)	Reaction component
10	10× BGD buffer
10	10× PLDβ/γ substrate
X	PLD protein source
Y	H_2O
100	Total reaction volume

2. Prepare a master mix for the appropriate number of reactions using the recipe above (*see* **Note 5**). The remainder of the protocol is the same as steps 3–7 from Subheading 3.1.

3.3 Oleate-Dependent PLD Assay

This assay can be used to detect PLDδ and PLDε classes. Other classes have no activity under these conditions (i.e., micromolar calcium, oleate, and no PIP_2).

1. A single reaction contains the following components.

1× (μl)	Reaction component
10	10× BGD buffer
10	10× PLDδ substrate
X	PLD protein source
Y	H_2O
100	Total reaction volume

2. Prepare a master mix for the appropriate number of reactions using the recipe above (*see* **Note 5**). The remainder of the protocol is the same as steps 3–7 from Subheading 3.1.

3.4 Ca^{2+}-Independent PLD Assay

This assay can be used to detect PLDζ enzymes. Other classes have no activity under these conditions (i.e., no calcium; *see* **Note 7**). EGTA (2 mM) may be added to reduce Ca^{2+} contamination in the assay.

1. A single reaction contains the following components.

1× (μl)	Reaction component
10	10× Z buffer
10	10× PLDζ substrate
X	PLD protein source
Y	H_2O
100	Total reaction volume

2. Prepare a master mix for the appropriate number of reactions using the recipe above (*see* **Note 5**). The remainder of the protocol is the same as steps 3–7 from Subheading 3.1.

3.5 Calculations

1. One microcurie (μCi) is equivalent to 2.22×10^6 dpm. Each reaction contains 0.02 μCi, so the maximum potential release of [^{3}H]choline is 0.02 μCi × 2.22×10^6 dpm/μCi = 44,400 dp m of radioactivity. A phosphatidylcholine conversion factor is calculated to convert cpm to dpm and to account for the total

volume of aqueous phase following the two-phase partitioning with chloroform and methanol (*see* **Note 8**):

$$\text{PC conversion factor} = (X\,\text{cpm} \times \text{counting efficiency} \times \text{volume correction})/$$
$$44{,}400\ \text{dpm} = (X\,\text{cpm} \times 2\ \text{dpm/cpm} \times 2.67)/44{,}400\ \text{dpm}.$$

2. PLD-specific activity (nmol/mg protein/min) is calculated by multiplying the PC conversion factor by the amount of non-radiolabeled PC (in nanomoles) and dividing by the amount of protein added (in milligrams) and duration of the assay (30 min).
3. The amount of PC per reaction, which differs in each of the four assay methods due to the differences in substrate composition, is 200, 2, 15, and 37 nmol for the assays described in Subheadings 3.1–3.4, respectively.
4. Example: If a sample gave a measurement of 4,000 cpm, its PC conversion factor would be

$$(4{,}000\ \text{cpm} \times 2\ \text{dpm/cpm} \times 2.67)/44{,}400\ \text{dpm} = 0.481.$$

If this result came from the oleate-dependent PLD assay (see Subheading 3.3) using 10 μg of protein, its specific activity would be

$$\text{Oleate-dependent PLD}(\text{nmol/mg/min}) = (\text{PC conversion factor})(\text{nmol PC})/(\text{mg protein})(30\,\text{min})$$
$$= (0.481)(15\ \text{nmol})/(0.01\ \text{mg})(30\,\text{min}) = 24\ \text{nmol/mg/min}.$$

4 Notes

1. It is convenient to purchase 100 mg of lyophilized egg yolk PC, add 1 ml of chloroform, remove an aliquot for substrate preparation, and store the rest at −20 °C in the original brown glass vial. To prevent oxidation of lipids during storage, wrap the rubber septum with parafilm and purge the headspace with N_2 delivered through a syringe needle with a second needle allowing purged air to escape the vial.
2. It is convenient to purchase 25 mg of egg PE powder, add 1 ml of chloroform, remove an aliquot for substrate preparation, and store the rest at −20 °C after purging with N_2.
3. It is convenient to purchase 5 mg of brain PIP_2 powder, add 1 ml of 20:9:1 chloroform:methanol:water, remove an aliquot for substrate preparation, and store the rest at −20 °C after purging with N_2.
4. Thoroughly remove the solvent under a gentle stream of N_2. After the solvent is completely evaporated, add 1 ml of deionized water. Sonicate just prior to use to emulsify the substrate

as follows: using a low-to-moderate-intensity sonicator setting, pulse (~1 s/pulse) the solution with the probe tip immersed in the liquid for 10 s followed by 10 s of resting on wet ice. Repeat this cycle until the milky/cloudy consistency becomes clear and no visible remnants of lipids appear stuck to the tube wall. Avoid frothing the solution during sonication (it gets easier with practice, but using a lower power setting and more cycles helps). Unused substrate can be stored at –20 °C but should be completely thawed and then sonicated before each use and a control without enzyme should be used to ensure that background degradation is low.

5. The volume of protein sample (denoted as X) can be varied to provide flexibility. We typically run triplicates of all experimental, control, and blank conditions. For the purpose of illustration, it is assumed that a protein sample volume of 15 μl will be used in all reactions. The volume of water needed per reaction (denoted as Y) in this example is therefore = 100 − 10 − 10 − 10 − 15 = 55. If 30 reactions are needed to account for all samples, blanks, and controls, it is advisable to prepare a master reaction mix for ~35 reactions by mixing the following components:

1× (μl)	35× (μl)	Reaction component
10	350	10× A buffer
10	350	10× PLDα substrate
10	350	5 mM SDS
15	–	PLD protein source
55	1,925	H_2O
100	2,975	

In this example, 85 μl (2,975 μl/35 reactions = 85 μl/reaction) of master mix should be transferred into individual reaction tubes. Assays for the other PLD classes are scaled up in an analogous fashion using the master mix recipes specified.

6. In practice, we choose to make a master mix omitting both the substrate and protein. After transferring the master mix and appropriate PLD protein samples to individual tubes, the reaction is initiated by adding the substrate. This approach works better when handling a large number of samples; allows for timely, synchronized assay initiation; and limits the chances for inadvertently mixing up samples.

7. To remove any residual calcium present in plant protein samples, one can supplement the PLDζ reaction mix with 2 mM EGTA to eliminate the possibility of background calcium-dependent PLD activity. In practice, we observe negligible levels of Ca^{2+}-dependent PLD when calcium is omitted from the reaction mix.

8. Tritium's (^3H) low energy makes its detection about 50 % efficient in most liquid scintillation counters, so a factor, 2 dpm/cpm, compensates for the difference between measured (cpm) and actual (dpm) radioactive decay. The aqueous volume comprises 100 μl of the reaction, 333 μl or 1/3 of 1 ml of 2:1 chloroform:methanol, and 100 μl of 2 M KCl for a total aqueous volume of 533 μl. A 200 μl aliquot is used for scintillation counting; thus a factor of 533/200 or 2.67 is needed to adjust for the radioactivity present in the total aqueous volume.

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