N-ACYLETHANOLAMINES AND PLANT PHOSPHOLIPASE D

THESIS

Presented to the Graduate Council of the

University of North Texas in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Shea Austin Brown, B.S.

Denton, Texas

December, 1998

Brown, Shea Austin, N-acylethanolamines and plant phospholipase D. Master of Science (Biochemistry), December, 1998, 70 pages, 2 tables, 12 illustrations, references, 72 titles.

Recently, three distinct isoforms of phospholipase D (PLD) were identified in *Arabidopsis thaliana*. PLD α represents the well-known form found in plants, while PLD β and γ have been only recently discovered (Pappan et al., 1997b; Qin et al., 1997). These isoforms differ in substrate selectivity and cofactors required for activity. Here, I report that PLD β and γ isoforms were active toward *N*-acylphosphatidylethanolamine (NAPE), but PLD α was not. The ability of PLD β and γ to hydrolyze NAPE marks a key difference from PLD α. *N*-acylethanolamines (NAE), the hydrolytic products of NAPE by PLD β and γ, inhibited PLD α from castor bean and cabbage. Inhibition of PLD α by NAE was dose-dependent and inversely proportional to acyl chain length and degree of unsaturation. Enzyme kinetic analysis suggested non-competitive inhibition of PLD α by NAE 14:0. In addition, a 1.2-kb tobacco (*Nicotiana tabacum L.)* cDNA fragment was isolated that possessed a 74% amino acid identity to *Arabidopsis* PLD β indicating that this isoform is expressed in tobacco cells. Collectively, these results provide evidence for NAE producing PLD activities and suggest a possible regulatory role for NAE with respect to PLD α.

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CHAPTER I

INTRODUCTION

Membranes are composed of a bilayer of amphipathic lipid molecules. This arrangement exposes the polar head group to the outer surface to allow interactions with water, different extrinsic proteins and other polar molecules. The molecular composition of biological membranes consists of proteins and polar lipids with a small amount of carbohydrates present as a constituent of glycoproteins or glycolipids. The percentages of these different molecules differ in different biological membranes, reflecting the different roles these membranes play in the physiology of the cell (Harwood, 1997). Therefore, lipids in biological membranes not only serve to maintain structural integrity of cells, but also to serve as an information mediator allowing communication between the cell and its environment. A well documented example is the phospholipase C/phosphoinositide signal transduction pathway (Munnick, 1998).

N-Acylphosphatidylethanolamine

N-Acylphosphatidylethanolamine (NAPE), a minor phospholipid, was first discovered in wheat flour (Bomstein, 1965). Now, NAPE appears to be a ubiquitous phospholipid found throughout the animal and plant kingdoms (Schmid, 1990; Chapman and Moore, 1993). NAPE has an unusual structure due to the presence of a third fatty acid moiety linked to the ethanolamine head group. NAPE is synthesized in plants by an enzyme designated NAPE synthase, which catalyzes the transfer of free fatty acids to the

primary amino group of phosphatidylethanolamine. This enzyme activity was CoA and ATP independent (Chapman and Moore, 1993), and was recently purified to homogeneity (Cai, et al., 1995; McAndrew and Chapman, 1998).

NAPE comprises about 2mol% of the total phospholipid in plant tissues (Chapman and Sprinkle, 1996; Sandoval et al., 1995). A physiological role for NAPE in plants has yet to be firmly established, but it was postulated to play a role in membrane stabilization under times of stress that result in threats to the integrity of the membrane bilayer (Schmid, 1990). This hypothesis was based upon biophysical studies with NAPE demonstrating that its unusual structural features allowed it to organize into a bilayer and function as a membrane stabilizing lipid(Akoka, 1988; LaFrance et al., 1997). In mammalian systems, NAPE accumulated in the membranes of ischemic heart and brain (Natarajan et al., 1986; Natarajan et al., 1982), but not in surrounding undamaged tissue. This suggests that NAPE was providing a protective role in membranes compromised by pathological stress.

More recently NAPE has been investigated as a precursor molecule for *N*-arachidonoylethanolamine (ananandamide, a type of NAE), a compound that binds to the cannabinoid receptor in mammalian neurons. Research involving NAE in mammalian systems has progressed rapidly in the last several years with identification of several other functional activities. NAEs are believed to play a role in such processes as a) inhibition of forskolin-mediated cAMP accumulation b) inhibition N-type Ca^{2+} channel activity c) sperm fertilizing capacity through inhibition of the acrosome reaction d) embryo implantation in the uterus (Schmid, 1996).

For plants, the role of NAPE and its catabolic product, NAE, is not known. New evidence has shown a six-fold increase in NAE released into tobacco cell culture medium after treatment with a fungal elicitor, xylanase(Chapman et al., 1998). The increase of NAEs was followed by an increase in the biosynthesis of NAPE by approximately 3-fold (Chapman et al., 1995). Furthermore, a microsomal phospholipase D was identified that catalyzed the conversion of NAPE to NAE, and evidence indicated this activity perhaps was regulated by activated G-proteins (Chapman et al., 1998; DeSouza, 1997).

Phospholipase D

Phospholipase D catalyzes the hydrolysis of phospholipids at the terminal phosphodiester bond, resulting in the formation of phosphatidic acid and the free head group. The enzyme was first discovered in plant carrot extracts in 1947 (Hanahan and Chaikoff, 1947) and has since been found in all organisms, making it a ubiquitous enzyme (Munnick, 1998). An interesting biochemical property useful in PLD analysis is its ability to catalyze a transphosphatidylation reaction using any primary alcohol, and producing the corresponding phosphatidyl-alchohol (PEOH) instead of phosphatidic acid (Singer, et al., 1997; Wang, 1997; Munnick, 1998).

Although the enzyme was discovered some 50 years ago, the physiological role is still being elucidated. A significant discovery was made recently in the identification of two additional isoforms in plants, PLD β and γ, which differ markedly from the most prevalent and well studied form of plant PLD, PLD α. PLD β and γ contain PIP_2-binding sites (Qin et al., 1997) and are catalytically active with micromolar

concentrations of calcium (Pappan et al., 1997b). These factors lead to the presumption that PLD β and γ are "highly regulated" isoforms.

Subcellular Localization

The majority of the studies to date examining subcelluar localization deal with the PLD α isoform. In discussing the occurrence of PLD within the cell and its physiological role, the reader should consider this information to be about PLDα unless otherwise stated. PLD has been found in both cytosolic and membrane bound fractions. The relative distribution of the two depends upon the tissue and developmental stage (Dyer et al., 1994; Ryu and Wang, 1995; Ryu and Wang, 1996; Wang, 1993) as well as the method of tissue homogenization. In young leaves, PLD activity was found mostly in soluble fractions, and immunocytochemical results showed that PLD was compartmentalized into the vacuoles and released upon homogenization of the leaves. This differed from mature leaves, where most of the PLD was associated with the plasma membrane and the endoplasmic reticulum(Xu et al., 1996). Other studies have shown translocation of PLD to the membrane upon wounding (Ryu and Wang, 1996). PLD β and γ activity was associated with membrane fractions, but the precise subcellular distribution of these isoforms has not been established (Pappan et al., 1997).

In mammalian systems, multiple PLD activities exist with a cytosolic form differing from a membrane bound form in substrate specificity, cofactor(s) requirements, and detergent effects. At this time it is unclear whether different PLD isoforms are expressed in the same cells or whether differences represent variant states of the same enzyme (Singer et al., 1997).

Catalytic Properties

PLD α purified from castor bean endosperm hydrolyzes PC, PE, and PG, but not PI or PS in single class phospholipid vesicles. PLD β and γ were only able to hydrolyze PE and PS in the presence of PIP_2. In the presence of PIP_2 and PE, PLD β and γ were able to hydrolyze PC, PG, and NAPE (Pappan et al., 1998). One of the most puzzling features of the predominant PLD α form is the requirement for millimolar (20-100) concentrations of calcium for maximal activity *in vitro*. This is in contrast to PLD β and γ enzyme activities which require micromolar (~50 μM) concentrations of calcium for optimal activity (Pappan et al.,1997) *in vitro*. The PLD activities in animals also are distinct; the cytosolic PLD can hydrolyze PE, PC, and PI but only in the presence of calcium. The two different mammalian membrane-associated PLDs are also distinguishable by the requirement of a PIP_2 for both, but one is stimulated by oleate and the other is stimulated by the small G-protein, ARF (Singer et al., 1997;Munnick et al.,1998).

Cloning and Expression

Sequencing of the N-terminus of the castor bean endosperm PLD allowed the first full length cDNA to be isolated. Since then, PLD cDNAs have been cloned from a number of plant species including maize (Accession No. D73410), rice (Accession No. D73411), black-eyed pea Accession No. U92656), *Pimpinella brachycarpa* (Accession No. U96438) and *Arabidopsis thaliana* (Accession No.U84568 and Accession No. AF027402) (Dyer, J.H., et al., 1995; Pappan, K. et al., 1997; Qun, W., et al., 1997; Ueki, J., et al., 1995; Morioka, S., et al., 1997). Three distinct PLD cDNAs were identified

from *Arabidopsis* and designated PLD α, β, and γ (Dyer et al., 1995; Pappan et al., 1997). There is a 73-90% amino acid sequence identity among the PLD α cDNAs from castor bean, rice, maize and *Arabidopsis*. This is in contrast to *Arabidopsis* PLD α protein having only 40% identity to *Arabidopsis* PLD β and γ; yet, PLD β and γ were reported to have 66% identity to each other at the amino acid sequence level (Pappan and Wang, 1998). It has been reported that PLD β is more closely related to the proteins cloned from yeast and humans than the α form (Wang, 1997). The *Arabidopsis* PLDα has a molecular mass of 91,800 daltons. Whereas, *Arabidopsis* β and γ reported molecular mass was 109,000 and 95,500 daltons, respectively (Pappan and Wang, 1998). Alignments of the plant PLD sequences have revealed three conserved regions. A calcium phospholipid binding domain (C2) was present in all plant PLDs (but is lacking in all mammalian and yeast PLDs to date) near the N-terminus of the sequence. Second, two putative catalytic HxKxxxD motifs have been identified in all PLDs cloned from plant, animals and yeast. It has been hypothesized that the absolute conservation of His, Lys and Asp residues at these positions suggest these residues are in the active site (Pointing, 1996; Sung, 1997). Third, a binding site for PIP_2 also was identified surrounding the second HKD motif. This region was rich in basic residues and has been reported to be responsible for polyphosphoinositide binding in proteins such as gelsolin and villin (Divecha and Irvine, 1995).

Role of PLD in Plants

Historically phospholipase D activity has been associated with large scale membrane degradation of lipids during germination and senescence (Munnick et al.,

1998). However, more recent studies suggest that in addition to membrane degradation, PLD may also have a more highly regulated role involving signal transduction (Munnick et al., 1995; Ryu and Wang, 1996; Young et al., 1996; Ritchie and Gilroy, 1998). High activity of the PLD enzyme was found in seeds undergoing germination, organs involved in senescence, and tissues susceptible to wounding or interacting with pathogens.

PLD has been suggested to play a role in seed germination. Immunological studies of PLD α showed an increase in protein levels in the endosperm tissue during germination (Wang et al., 1993). In rice, an increase in mRNA levels for PLD α was also reported shortly after imbibition of the seedlings (Ueki et al., 1995). Another study, reported three variant forms of PLD α in soybean, the expression level of these 3 proteins increased during germination (Dyer et al., 1994). More recently, a study conducted in barley reported PLD activity in the aleurone may be involved in signal transduction events which lead to the triggering of abscisic acid (ABA) response involved in seed germination in barley aluerone (Ritchie and Gilroy, 1998).

PLD-mediated hydrolysis of phospholipids leading to membrane deterioration has been proposed in senescing plants (Pappan and Wang, 1998). PLD involvement in senescence is of particular interest due to the monetary value associated with the spoilage of agricultural crops (Pappan and Wang, 1998). A recent study used PLD α- antisense suppressed *Arabidopsis* plants to investigate the role of PLD α in plant senescence. This study provided direct evidence of PLD α involvement in ABA- and ethylene- promoted senescence in detached leaves. However, detached leaves from wild-type and PLD α suppressed plants in the absence of ABA or ethylene were shown to have a similar rate of

growth and development, which included the rate of senescence (Fan et al., 1997). Another study, examined the regulation of tomato fruit ripening and its relationship to PLD α activity; the researchers drew a similar conclusion as Fan et al. (1997).

Wounding of plants occurs in nature usually when an herbivore feeds on plant tissue. Changes in PLD activity have been described in relation to wounding (Wang, 1996). PLD α activity was shown to increase in wounded castor bean leaves. This activity was not due to an increase in protein expression, but rather a translocation of the PLD α from the cytosol to the membranes in both wounded and unwounded cells. A similar translocation pattern of PLD α also was obtained by an increase in free calcium at physiological concentrations in the homogenization buffer (Ryu and Wang, 1996).

Perception of pathogens by a plant leads to a hypersensitive response (HR) cascade which results in membrane damage and cell collapse (Goodman and Nocacky, 1994). Rice leaves undergoing interactions with pathogens, revealed an increase in PLD α mRNA transcripts, and a change in the distribution of the PLD protein in the membranes. The PLD protein, in resistant interactions, was clustered in plasma membranes at the site of pathogen attack; however, in susceptible interactions the PLD protein was distributed uniformly along the plasma membrane (Young et al., 1996). This study provided evidence for the role of PLD in defense to pathogen invasion. A recent study suggested the involvement of PLD in elicitor-treated tobacco cells, where NAPE was hydrolyzed to NAE. Evidence showed the accumulation of two NAE species, N-lauroyl- and N-myristoyl-ethanolamine, and a mastoparan-stimulated PLD activity detected in microsomes that hydrolyzed NAPE to NAE. This latest evidence leads to

questions involving the role of PLD activity toward NAPE and the physiological significance of the NAE in plant defense reponses.

Research Rationale and Objectives

Hydrolysis of NAPE by PLD in plants and animals prompted the investigation of determining which of the newly discovered PLD isoforms were responsible for this hydrolysis. This investigation was possible due to the expression of each of the isoforms in *E. coli.* It was found that two different isoforms, PLD β and γ, were capable of hydrolysis of NAPE. NAE, the product of NAPE hydrolysis, was examined for its biological effects on PLD α activity. This was due, in part, to evidence that PLD α , the most prevalent form of PLD in plants, was not shown to have activity toward NAPE. Other rationale included the possibility of crosstalk between the PLD isoforms. Tobacco PLD β cDNA was isolated to further establish the role of NAE's physiological significance in plants.

CHAPTER II

MATERIALS AND METHODS

Chemicals

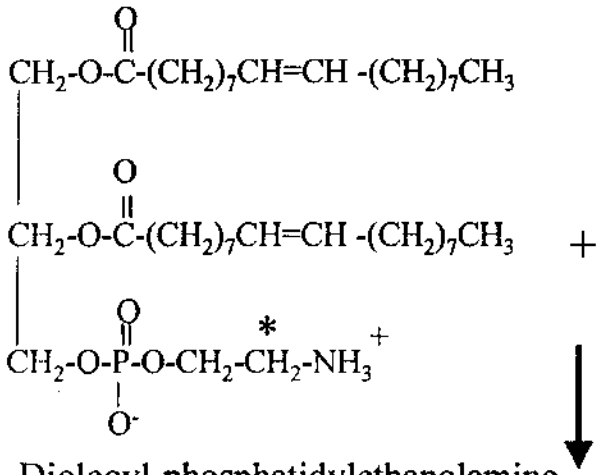
L-3-Phosphatidyl [2-^{14}C] ethanolamine, 1,2-dioleoyl purchased from Amersham Life Sciences (Elk Grove, IL). Dioleolyl-[2^{14}C -oleoyl]glycero-3-P-choline was purchased from Dupont, NEN Life Sciences. Phosphatidylcholine, polyphosphatidylinositol-bisphosphate (PIP_2), phosphatidylethanolamine(dioleoyl), palmitoyl chloride, ampicillin, phenylmethylsulfonyl fluoride, cabbage PLD Type V, *Streptomyces chromofuscus* PLD, bovine serum albumin, coomasssie brilliant blue, IPTG were from Sigma Chemical Co. (St. Louis, MO). All other reagents used in biochemical analysis were purchased from Fisher Scientific (Pittsburg, PA), unless otherwise specified.

Chemical Synthesis of NAPE

Chemical synthesis of radiolabeled NAPE was modified from a reaction scheme originally proposed by Dawson (1969). A 10-fold increase in phosphatidylethanolamine concentration forced the reaction toward completion. The modification led to a three-fold greater conversion of product (Figure 1) than previously reported (DeSouza, 1997).

[^{14}C]NAPE was synthesized chemically using 1 μCi of *sn*-1,-2 dioleoyl-glycero-3-phosphoryl-[ethanolamine-2-^{14}C] ethanolamine (54mCi/mmol), 2.7 μmol of

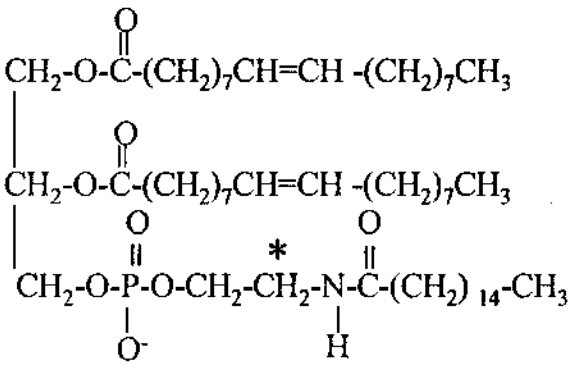
Figure 1. Chemical synthesis of NAPE (A) Schematic of chemical synthesis of NAPE from dioleoyl-phosphatidylethanolamine and palmitoyl chloride and (B) Chromatogram of radiometric scan of NAPE (Bioscan system 200 Imaging Scanner), synthesized and isolated by thin-layer chromatography.



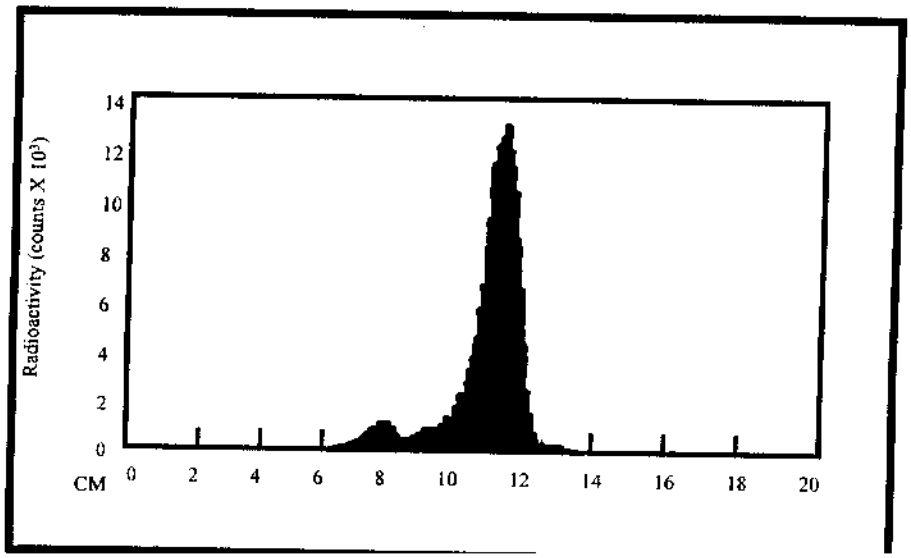
Dioleoyl-phosphatidylethanolamine

+ $ClOC-(CH_2)_{14}-CH_3$

Palmitoyl chloride



N-palmitoyl dioleoyl-phosphatidylethamolamine
(NAPE)



nonradiolabeled dioleoyl-PE, and 8.3 μmol of palmitoyl chloride as previously described (Dawson, et al., 1969)

NAPE was separated from PE by silica-gel thin-layer chromatography (conditions described below), recovered in chloroform, and quantified by liquid scintillation counting. This procedure had a routine conversion from PE to NAPE of 90% or greater.

Expression of active PLDs in *E. coli*

The recombinant castor bean PLD α and *Arabidopsis* PLD β, γ and δ in pBluescript SK(-) were provided by Dr. X. Wang (Kansas State University, Manhattan, KA). The following protocol was adapted from previously described methods (Pappan, et al., 1997 b). Expression of PLD α, β, γ and δ from their cDNAs was performed using pBluescript SK(-) containing the cDNA inserts in *E. coli* (JM109) cells. Fifty microliters of an overnight culture containing the transformed JM109 cells were added to 25 mL of LB medium with 50 μg/mL ampicillin. The cells were incubated at 37°C with shaking for 3 h, and then IPTG was added to a final concentration of 2 mM. The cells were grown overnight at 30°C and pelleted by centrifugation (2000 rpm, 10 min). The cells were then resuspended in an assay mixture containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.25 mM phenmethylsulfonyl fluoride, 2 mM EDTA and then pelleted by centrifugation (2000 rpm, 10 min). The cells were lysed by sonication in the resuspension buffer and cell debris was removed by centrifugation at 10,000xg for 5 min. Supernatants were used for assays of enzyme activity and protein content.

PLD Activity Assays and NAE Inhibition Studies

PLD isoforms were assayed under two different sets of conditions. These conditions were previously determined to yield optimal activity *in vitro* (Pappan et al., 1997 and Pappan et al., 1998).

PIP_2-Dependent Assay

The basic assay mixture contained 100 mM Mes buffer (pH 7.0), 50 μM $CaCl_2$, 80 mM KCl, 0.4 mM lipid vesicles, and 20 μg of protein from *E.coli* lysates in a total volume of 150 μL PLD activity toward PC was assayed in the presence of 1% ethanol (v/v). Lipid vesicles for PC hydrolysis included 16 nmol dioleoyl-[oleoyl-2^{14}C] glycero-3-P-choline (0.05μCi) PC, 112 nmol dioleoyl PE, 6 nmol PIP_2. Lipid vesicles for NAPE hydrolysis were composed of 16 nmol [^{14}C]NAPE (0.006 μCi), 112 nmol dioleoyl PE, and 6 nmol of PIP_2. PLD activity was measured and evaluated by radiometric scanning of TLC separation of lipid soluble reaction products.

High Calcium, PIP_2-Independent Assay

The basic assay mixture contained 100 mM Mes buffer (pH 6.5), 25 mM $CaCl_2$, 0.5 mM SDS, 0.4 mM lipid vesicles containing PC or NAPE:PC (1:1 molar ratio), and 20 μg of protein. Hydrolytic activity was assessed by measuring the production of radiolabeled phosphatidylethanol or *N*-acylethanolamine.

Control assays were performed using 20 μg of protein from lysed bacteria minus IPTG. The reaction was initiated by the addition of enzyme and proceeded for 30 min at 30°C in a shaking water bath (120 rpm). Reactions were stopped by addition of 2 mL boiling isopropanol.

NAE Inhibition of Phospholipase D α

Activity assays were conducted with castor bean, cabbage, and *Streptomyces chromofuscus* PLD in the presence of different concentration and species of NAE to examine possible effects on enzyme activity. The assay mixture contained the above components as mentioned for high calcium, PIP_2-independent assay mixture with a modification of 2 mM PC and NAE (12:0-18:3) at concentrations as noted in text. Enzyme (20 μg for castor bean and 0.5 and 1 unit for cabbage and *Streptomyces chromofuscus*, respectively) was added and reaction allowed to proceed at 15 or 30 min in a shaking water bath (120 rpm) at 30°C. Reactions were halted by the addition of 2 mL boiling isopropanol.

N-acylethanolamines were synthesized by the addition of 25 mg of acyl chloride in 2.5 mL of dichloromethane to 2.5mL of ethanolamine. Reaction was allowed to proceed for 15 min at room temperature with gentle swirling. The reaction was stopped by the addition of 10 mL of ultrapure water. The organic layer was washed an additional two times with 10 mL of ultrapure water. Samples were dried under a stream of N_2, weighed and resuspended in methanol. (NAEs were synthesized by Dr. K Chapman and R. Blair.) NAE yield and purity were determined by GC-MS.

Lipid Extraction

Lipid extractions from assay reactions were based on previously described methods by Chapman and Moore (1993). Hot isopropanol (70°C) was routinely used (2 mL) to inactivate the enzyme following assays. The alcohol/aqueous mixture was then allowed to cool before adding chloroform (1 mL) for extraction for 1 hour (or overnight

at 4°C). Mixtures were partitioned by the addition of 2 mL 1M KCl and 1mL $CHCl_3$. Centrifugation at 2000 rpm for 5 min (Beckman TJ-6 centrifuge) facilitated partitioning of the two phases. The aqueous phase was aspirated off and the chloroform layer was washed two more times with 2 mL 1 M KCl. The chloroform/ lipid mixture remaining was transferred to 3 mL scintillation vials (Fisher) and evaporated to dryness under N_2. The lipids were resuspended in 50 µL of chloroform: methanol (2:1) and analyzed by thin-layer chromatography and radiometric scanning.

Thin Layer Chromatography

Lipid separation was performed using one dimensional TLC. The lipid samples (50 µL) were applied to 20x20 cm silica gel G plates (Whatman, layer thickness 250 µM). NAPE/NAE separation was conducted first in hexane: diethylether (80:20; 45 min) then into subsequent chamber containing chloroform: methanol: water (80:35:1; 60 min) in the same direction. PC/ Phosphatidylethanol separation was performed in chloroform: methanol: ammonium hydroxide (65:35:5; 55 min) . The lipids were visualized by a brief exposure to iodine vapor. Radiolabeled product was quantified as a percentage of the total radioactive lipid by radiometric scanning (Bioscan system 200 Imaging Scanner, Bioscan, Washington, D.C.). Enzyme activity was calculated based on the radiospecific activity of substrate.

Estimation of Protein Content

Protein content was estimated by the Bradford method (1976) using bovine serum albumin as the protein standard. One milliliter of Bradford reagent (0.117 mM Coomassie Blue G; 0.85% phosphoric acid; 4.75% ethanol) was added to a mixture

containing 2-50 μL of protein sample and 50 μL of 1 N NaOH. The mixtures were allowed to stand for 5 min before measuring absorbance at 595 nm in a Milton Roy spectrophotometer against appropriate blanks.

Degenerate Primer Design

Degenerate oligonucleotide primers were synthesized and purified by Bio-Synthesis, Inc. (Lewisville, TX). Primers were based on the amino acid sequences from *Arabidopsis thaliana* PLD isoforms β and γ (Qin et. al., 1997). Table I provides a list of the primers along with the predicted T_m and the degree of degeneracy for each.

Amplification of cDNA by PCR

A tobacco cDNA library (provided by Dr. G. An, Washington State University) was constructed from mRNA isolated from tobacco NT-1 (*Nicotiana tabacum L.*) cell line in early exponential growth phase. Double stranded cDNA was packaged into the lambda ZAPII library (Stratagene) with external EcoRI and NotI linkers (Pharmacia). The average insert size in the cDNA library was 1kb.

Two microliters of the supernatant containing the bacteriophage of the tobacco cDNA library in SM buffer (5.8 g of NaCl, 2.0 g of $MgSO_4$-H_2O, 50.0 mL of 1 M Tris-HCl pH 7.5, 5.0 mL of 2% w/v gelatin) was used as template for PCR reactions. The supernatant was heated for 5min at 70°C to lyse phage heads, cooled at 4°C for 5 min, and added to the reaction mixture for a final volume of 50μL. The PCR reaction mixture contained 10 mM Tris-HCl (pH8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 1.25 units of AmpliTaq Gold™ DNA polmerase (Perkin-Elmer) and 1μM each of the forward and reverse primers. A "hot

Table I. Degenerate Oligonucleotide Primers for PCR and RT-PCR experiments

Primer	Amino acid sequence[a] Nucleotide sequence[b]	Length (nt)	T_m[c] (°C)	Degeneracy (n-fold)
-[d]1	ggQHKTIEMM 5' catcatytcdatngtytt rtgytgcc3'	26	70	192
+2	IYTHHEKac 5'athtayacncayc aygaraarac3'	23	54	384
-3	cnIYTHHEKac 5'gtyttytcrtgrtgngtr tadatng3'	25	63	1536
+4	ECWFWCgg 5'gartgytggttyt ggtgygg3'	20	67	16
+5	HGKCWEDM 5'cayggnaartgytgg gargayatg3'	24	68	128
-6	EEPENMECg 5'crcaytccatrttyt cnggytcytc3'	25	69	256

Degenerate primer sequences were designed from amino acid sequences of *Arabidopsis* PLD β and γ. Synthesis and purification was performed by Bio-Synthesis, Lewisville, TX. [a] Amino acid sequences are capitalized. [b]Nucleotide sequences are in lower case. Symbols used to denote multiple sequences are as follows: y=c+t; r=a+g; d=g+a+t; n=a+c+g+t. [c]The T_m's were calculated using "DNA Synthesis Oligo Calculator" (ž HYPERLINK http://www.biotech.ufl.edu/cgi-bin/doa.cgi/) – http://www.biotech.ufl.edu/cgi-bin/doa.cgi/) . [d]The + and – signs indicate the coding strand or complimentary to the coding strand, respectively.

start" method was implemented according to manufacturers instructions (Perkin-Elmer) followed by amplification in a thermal cycler (Perkin-Elmer Model 2400) . The amplification was performed for 35 cycles with 30 sec at 94°C, 30 sec at 55°C-65°C (annealing temperatures were varied to optimize PCR product) and 1 min at 72°C. After the last cycle, the amplification was extended for 10 min at 72°C. PCR amplification products were electrophoresed in 3% agarose gels (PCR, Perkin-Elmer) in standard TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) for 2.5 h at 70 V. A 1.2-kb PCR product was purified from agarose gels using a Prep-a-Gene™ DNA purification kit (Bio-Rad), according to manufacturer's instructions.

Amplification of cDNA by RT-PCR

Total RNA was extracted from tobacco KY-14 *(Nicotiana tabacum L.)* cell line and from various cotton organs (cotyledons, hypocotyls, roots, leaves and embryos) by the modified hot borate method of Wan and Wilkins (1994). Total RNA (0.2 μg/μL) was used for first-strand cDNA synthesis with 5 units of Avian myeloblastosis virus (AMV) reverse transcriptase. First-strand synthesis along with the subsequent amplification cycles were carried out using Access RT-PCR System (Promega). First-strand synthesis was carried out at 48°C for 45 min (Perkin-Elmer thermal cycler 2400). Followed by 2 min at 94°C to inactivate the AMV reverse transcriptase, and 40 cycles of amplification. The amplification cycles were carried out as described in the previous section. The final round of amplication was followed by a 7 min extension at 72°C.

Subcloning of PCR Products

PCR products (in 5 µL) were reamplified in a 50 µL reaction mixture containing 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 , 10 µg/mL nuclease-free BSA, 2.5 units of *Pfu* DNA polymerase, 0.2 mM total of dATP, dTTP, dGTP, dCTP and 0.5µM of the same primers used in original amplification. The reaction was incubated for 45 sec at 95°C, then followed by 35 cycles of amplification with 30 sec at 95° C, 30 sec at 55°C-65°C (annealing temperature was identical to original PCR amplification), 2.5 min at 72°C. After the last cycle, the amplification was extended for 10 min at 72°C. The PCR reaction product was immediately purified from the reaction mixture using the Prep-A-Gene DNA purification kit (as above). An aliquot (5 µL) was quantified in a 3% agarose gel with DNA mass markers (10-200 ng/band, corresponding to 100-2000bp, respectively; GIBCO) stained overnight with ethidium bromide.

Purified PCR products were subcloned (blunt-end ligation) into pZErO-2.1™(Invitrogen) digested with *Eco*RV (Promega)(5:1 PCR product: vector ratio) according to manufacturer's instructions using Fast-Link DNA ligation kit (Epicentre Technologies). One Shot™ Top 10 *E.coli* cells (Invitrogen) were transformed with ligated plasmids. The *E. coli* cells were made competent by cell suspension in 0.1M calcium chloride and 0.01M rubidium chloride (Seidman et al., 1997). The ligation/transformation mixture was plated on selection media containing NZY (21 mg/ml), 0.05 mg/ml kanamycin, and 1 mM IPTG. The pZErO-2.1(vector contains a lethal gene *ccd*B (control of cell death) induced by the lacZ promoter. When an insert is

present disruption of the lacZ promoter occurs, thereby allowing these cells to be viable. Plasmid DNA was isolated (Wizard Plus SV miniprep DNA purification kit, Promega) from 10 mL cultures selected from a single colony and grown overnight in NZY under selection pressure of kanamycin (concentrations as stated previously) until late log phase. The plasmid DNA was digested with *Xba*I and *Sac*I and separated on a 1.5% agarose gel to verify PCR inserts. DNA quantity and purity were estimated spectrophotometrically by recording absorbance at 260 and 280 nm.

DNA sequencing and analysis

PCR fragments subcloned into pZErO-2.1™ plasmids were sequenced using IRD-41 labeled M13 Forward and Reverse primers (LI-COR), using a Sequi-Therm EXCEL II Kit-LC fluorescent-labeled primer. The dideoxy-chain termination method was carried out according to manufacturers instructions (Epicentre Technologies), and processed on an automated LI-COR Sequencer (LI-COR Inc., Lincoln, NE, Model 4000). Analysis of nucleotide and amino acid sequences were performed using DNASIS software (HIBIO DNASIS for Windows, version 2) and BLAST programs (Altschul et al., 1990).

CHAPTER III

RESULTS

Phospholipase D Activity toward NAPE and PC among isoforms

The activities of PLD isoforms expressed in *E. coli* cells were compared (Figure 2). The formation of radiolabeled phosphatidylalcohol was used to measure PLD activity when radiolabeled PC was used as a substrate. Radiolabeled NAE formation was used to measure PLD activity when radiolabeled NAPE was used as a substrate. NAPE hydrolysis was observed for both PLD β and γ, whereas PLD α and δ hydrolysis of NAPE was not detected. PLD β showed comparable hydrolytic activity toward NAPE and PC; whereas, PLD γ activity toward NAPE was three times greater than toward PC (Figure 2). NAPE and PC hydrolysis occurred in lipid vesicles containing PIP_2, 50 μM Ca^{2+}, and over 50% PE. No activity was observed for PLD α in lipid vesicles composed of NAPE alone or NAPE:PC (1:1 molar ratio) (Figure 2B).The structure of NAPE and the site of cleavage by PLD β and γ are shown in figure 3. This study demonstrates that PLD β and γ , which are known to be different from the conventional PLD α (Pappan et al., 1998), are capable of hydrolyzing NAPE.

In addition to radiometric scanning for detection of product, autoradiograms were produced for qualitative comparison. Thin-layer chromatography separation of radiolabeled NAPE (Figure 2C) or PC (Figure 2D) showed hydrolysis

and/or transphosphatidylation, respectively, by the PLD isoforms expressed in *E. coli* and *Streptomyces chromofuscus* PLD. Hydrolysis or transphosphatidylation of radiolabeled NAPE and PC, respectively, by PLD β and γ was visualized by exposure to x-ray film for approximately 3 days. The formation of NAE was visually detected on autoradiograms of TLC plates for PLD β and γ, but was not for PLD α. In a positive control, *Streptomyces chromofuscus* hydrolyzed NAPE to NAE as documented previously (Schmid et al., 1990). Both PLD β and γ were able to produce phosphatidylethanol from PC (Figure 2D, Lanes 2 and 4).

NAE effects on PLD α activity

NAE inhibited the activity of castor bean PLD α expressed in *E. coli* cells (Figure 4). Castor bean PLD α activity was assayed in a high Ca^{2+} dependent assay mixture that contained radiolabeled PC and ethanol as substrates and quantified radiolabeled phosphatidylethanol as product. All NAEs were effective inhibitors at high concentrations (200 μM), similar results were reported for lysophosphatidylethanolamine (Ryu et al., 1997). In general the long chain, unsaturated *N*-acylethanolamines demonstrated less inhibitory effects of castor bean PLD α. Using medium to high concentrations (50-200 μM) of NAE 12:0 and NAE 14:0, the castor bean PLD α showed no activity (not shown). Submicromolar to low micromolar concentrations of NAE were tested for their inhibitory effectiveness against castor bean PLD α as shown in Figure 5. Both NAEs were effective inhibitors at low concentrations.

To determine the extent of *N*-acylethanolamine inhibition on phospholipase D from different species, we studied the effect of NAE on highly purified cabbage PLD α and *Streptomyces chromofuscus* PLD. Figure 6 shows NAE 12:0 and NAE 14:0 at submicromolar to low micromolar amounts were effective inhibitors of cabbage PLD α, but not of *Streptomyces chromofuscus* PLD. PLD activity for *Streptomyces chromofuscus* PLD was measured by the production of radiolabeled phosphatidic acid. The cabbage PLD activity was 37% and 22% of the control for NAE 12:0 and NAE 14:0, respectively, at a concentration of 1 μM. NAE 14:0 appeared to have a somewhat greater inhibitory effect toward cabbage PLD α than NAE 12:0. Cabbage PLD activity was unchanged in the presence of 10 μM myristic acid (14:0,data not shown). Other workers have demonstrated ethanolamine, the head group of NAE, had no inhibitory effect on PLD (Ryu et al., 1997), indicating structural specificity for the NAE-type molecule.

Increasing substrate concentration (PC) on cabbage PLD was analyzed in the presence and absence of NAE to characterize the type of inhibition of PLD (Figure 7). The apparent Vmax for cabbage PLD in the absence and presence of 0.01 μM NAE 14:0 was 16.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein and 11.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively. The apparent Km of 2.5 mM for cabbage PLD was unchanged in the presence of NAE 14:0. These results suggest noncompetitive inhibition of cabbage PLD by NAE 14:0, with an apparent K_I for NAE 14:0 of 0.02 μM.

Table II summarizes the IC_{50} values for all of the NAEs tested on castor bean PLD α. Inhibitor concentration of the different NAE species resulting in 50% of the

maximal activity ranged from 0.1 μM for NAE 14:0 to 80 μM for NAE 18:3, this accounts for an 800-fold difference in inhibitory effects of the different NAE species. Together these results clearly demonstrate that NAE (especially NAE 12:0 and NAE 14:0) have an inhibitory effect on plant PLD α. NAE inhibition of PLD α raises the possibility that of NAE acts as a lipid mediator in vivo to regulate PLD α activity. Although *Arabidopsis* PLD β and γ were not tested for activity in the presence of NAEs, the NAE concentration when hydrolyzed by the PLD β and γ was approximately 10 μM.

Isolation and sequence comparison of a putative phospholipase D β from tobacco

To better understand the role of PLD in NAPE metabolism, molecular analysis was planned to determine if PLD β or γ were present in tobacco cell suspensions. A pair of degenerate PCR primers were designed based on the amino acid sequences of *Arabidopsis* PLD β (Pappan et al., 1997) and γ (Qin et al., 1997). The tobacco cDNA library was constructed from mRNA isolated from tobacco (*Nicotiana tabacum* L.) NT-1 cell line in early exponential growth phase (gift of Dr. G. An, Washington State University). A cDNA fragment was amplified from a tobacco cDNA library with a degenerate primer combination (Table I, Primer -1 and +5) using PCR. The PCR fragment was estimated to be 1.2-kb. Subcloning was performed for sequencing analysis using pZErO-2.1™ as the vector. Many recombinant clones in *E. coli* Top 10™ cells were obtained and four were randomly chosen for plasmid DNA isolation and restriction digestion(Figure 8). Vector DNA was digested with restriction enzymes *Sac* I and *Xba* I. An insert was detected in two of the samples, denoted clone II1 and clone II6 (Figure 8, lanes10 and 13). Clone II6 sequence was identical to that of II1. To completely

characterize the PCR product, clone II1 was digested with enzymes at the multiple cloning site of the pZErO-2.1™ vector. Six different enzymes were incubated with clone II1 and electrophoresed in a 1.5% agarose gel (Figure 9). *Hin*d III cleaved the PCR fragment at an internal site (Figure 9, lane 2). Both the small and the large fragment of clone II1 from *Hin*d III digestion were subcloned and sequenced. Figure 10 is a physical map of clone II1 showing the internal restriction site for *Hin*d III, the multiple cloning site, and the direction of the M13 forward and reverse primers used in the sequencing reactions.

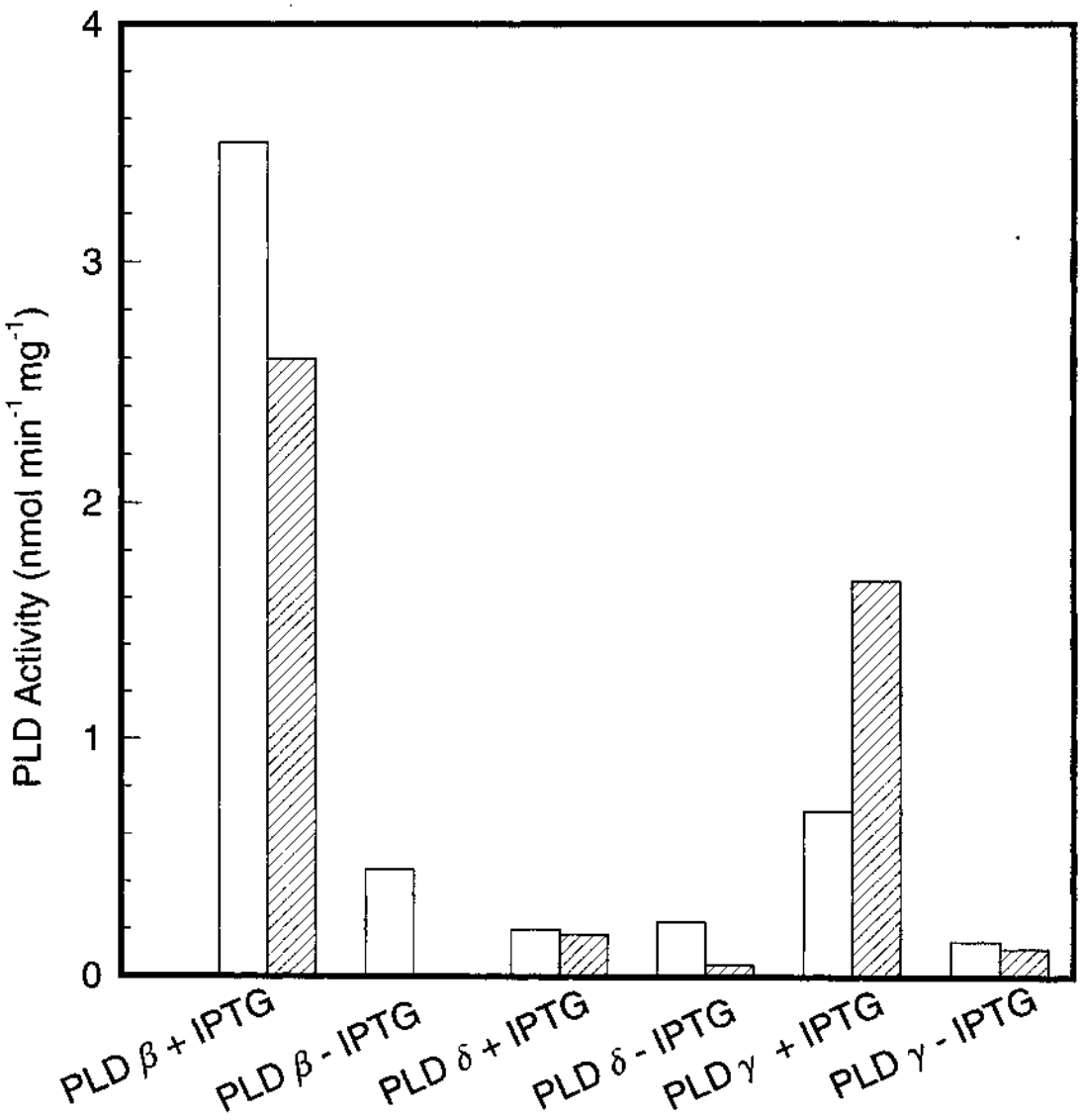
The cDNA fragments in pZErO-2.1™ vector designated II1 and subfragment thereof, were sequenced on both strands using M13 forward and reverse primers. The cDNA fragment was 1170-bp, and the deduced amino acid sequence is shown below the nucleotide sequence in Figure 11. The degenerate primers used to generate the sequence were found on the 5' and the 3' end of the fragment, as indicated by the dashed arrow lines. Included within this 390 amino acid segment was one putative catalytic domains, denoted HXKXXXXD. Using the BLAST program (Altschul et al., 1990) clone II1 had highest homology to *Arabidopsis* PLD β (Accession No. U84568), followed by *Arabidopsis* PLD γ (Accession No. AF02408) over the length of the 1.2-kb fragment.

The deduced amino acid sequence for the putative tobacco PLD β clone (II1) was aligned with amino acid sequences for *Arabidopsis* PLD β, *Arabidopsis* PLD γ and tobacco PLD α (Accession No. Z84822) (Figure 12). Alignment of the putative tobacco PLD β clone (II1) and *Arabidopsis* PLD β indicates a 74% identity between the two, while 65% and 52% identities were revealed with alignment of the tobacco clone II1

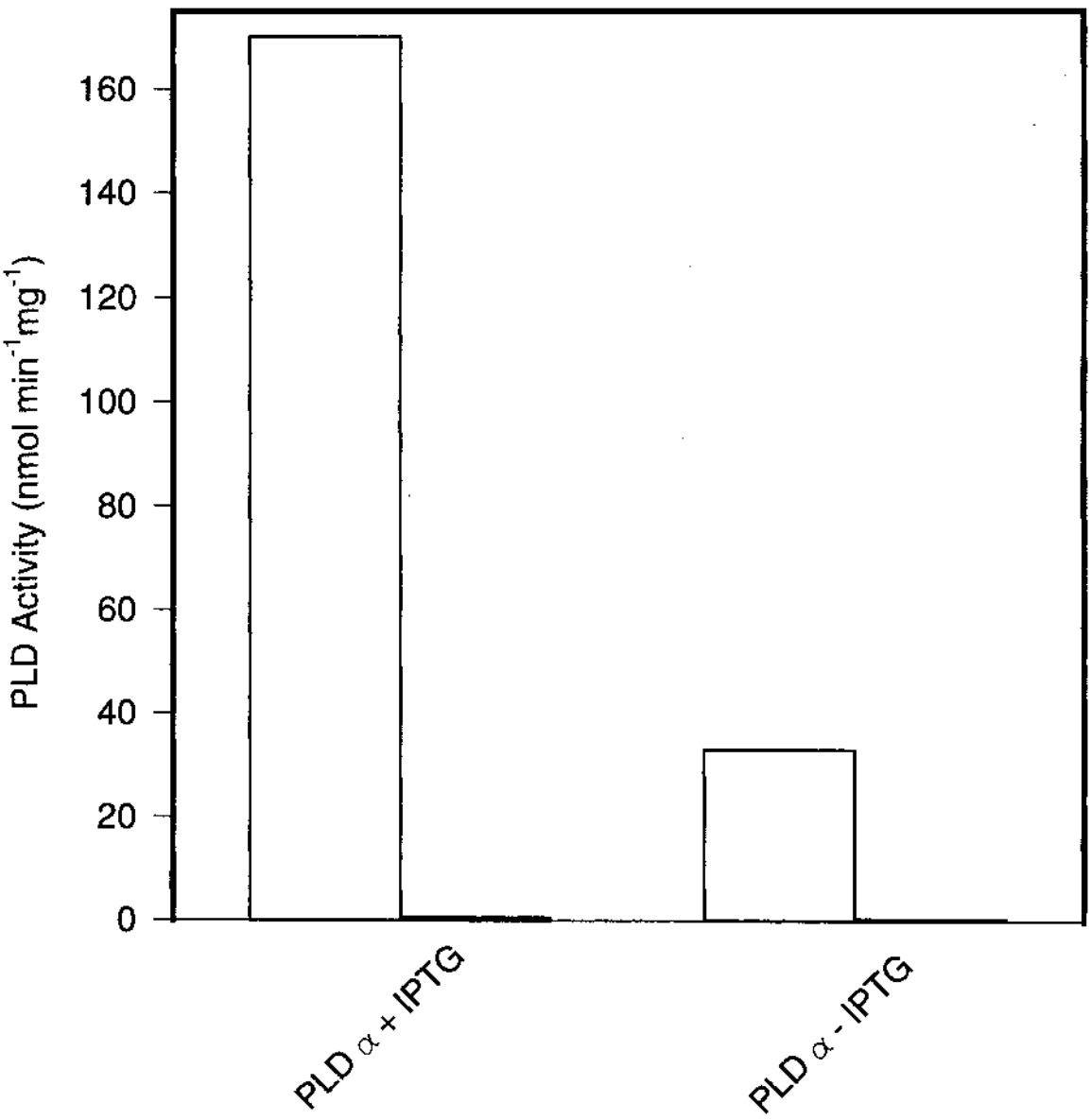
with *Arabidopsis* PLD γ and tobacco PLD α, respectively. Based upon the sequence similarity, it is likely that clone H1 encodes a portion of the PLD β expressed in tobacco cells.

Figure 2. Comparison of enzymatic activities of PLD α, β, δ and γ expressed in *E. coli* toward PC (open bars) and NAPE (hatched bars). (A) PLD β, δ and γ activities were assayed in lipid vesicles composed of 85.2 mol% PE, 6.6 mol% PIP_2, and 8.2 mol% NAPE or PC with 50 μM Ca^{2+}. Protein (20 μg) from *E. coli* lysates was added to initiate the reaction. Activity toward PC was measured by the formation of [^{14}C]acyl-PEOH(□). NAPE hydrolysis was measured by the formation of N-acyl-[^{14}C]ethanolamine (▨). Multiple experiments showed similar trends. Values are means of one representative experiment with replicate samples. (B) PLD α activity measured in single class vesicles of NAPE or PC, 25 mM Ca^{2+}, and 0.5 mM SDS. Protein (20 μg) from *E. coli* lysates was added to initiate the reaction. PLD activity toward PC was measured by the production of [^{14}C]acyl-PEOH and that toward NAPE hydrolysis was measured by release of N-acyl-[^{14}C]ethanolamine from ^{14}C-NAPE. Data are representative of multiple experiments exhibiting similar trends. (C) Autoradiogram of a TLC plate separating lipid soluble reaction products from PLD α, β and γ assays. Lanes 1, 3, and 5 are PLD β, γ, and α following induction of PLD expression with IPTG (12 h). Lanes 2, 4, and 6 are PLD β, γ, and α without induction. Lanes 7 and 8 are without and with *Streptomyces chromofuscus* PLD and show the relative positions of NAPE and NAE. (D) Autoradiogram of a TLC plate separating lipid soluble reaction products from PLD α, β and γ assays. Lane 1 is negative control (PC alone). Lane 2 and 4 are PLD β and γ following induction of PLD expression with IPTG (12 h). Lanes 3 and 5 are PLD β and γ, without induction.

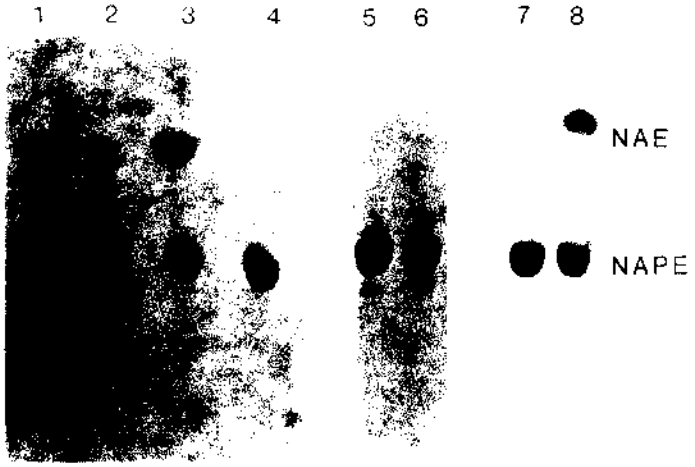
A


PLD Activity (nmol min-1 mg-1)
4
3
2
1
0
PLD β + IPTG
PLD β - IPTG
PLD δ + IPTG
PLD δ - IPTG
PLD γ + IPTG
PLD γ - IPTG

B


PLD Activity (nmol min-1mg-1)
160
140
120
100
80
60
40
20
0
PLD α + IPTG
PLD α - IPTG

C



D

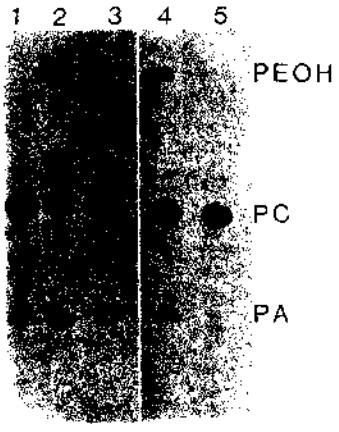


Figure 3. Structure of NAPE and site of cleavage by PLD

sn-1,2-dipalmitoyl phosphatidyl (*N*-palmitoyl) ethanolamine (an NAPE)

Products: *sn*-1,2-dipalmitoyl phosphatidate (a PA) and *N*-palmitoylethanolamine (an NAE)

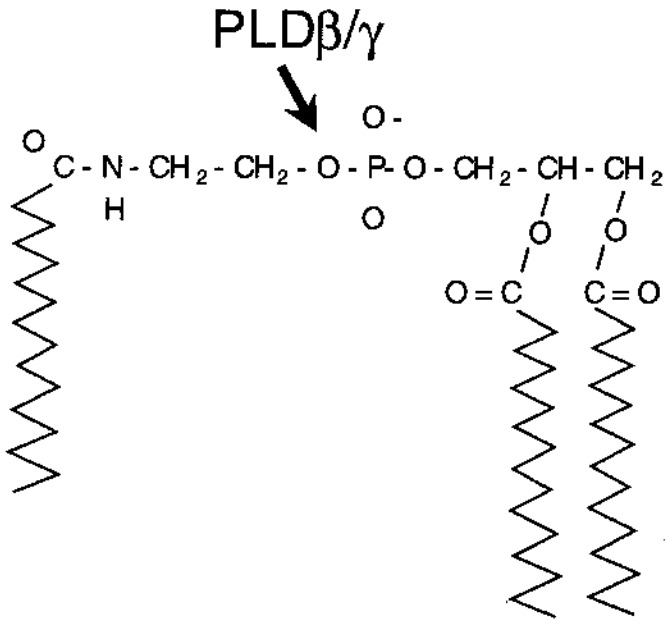


Figure 4. Inhibition of castor bean PLD α activity (expressed in *E. coli*) by increasing concentrations of NAE 16:0 (●), NAE 18:0 (■), NAE 18:1 (▲), NAE 18:2 (▼), NAE 18:3 (◆). Protein (20 μg) was added to initiate the reaction. Assays were conducted as described in Figure 2. Data are from one experiment. Similar results were obtained in repeated experiments.

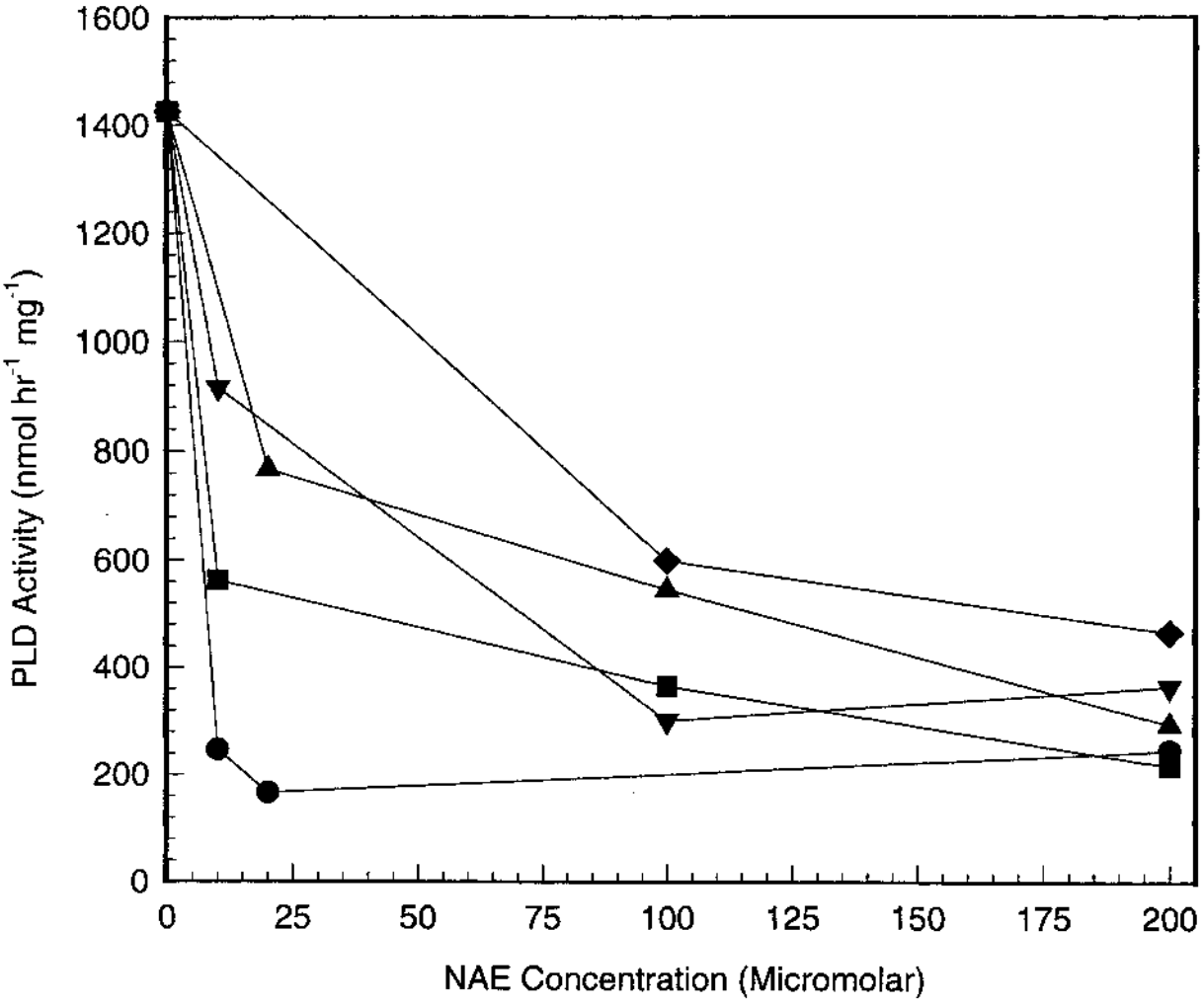

1600
1400
1200
1000
800
600
400
200
0
PLD Activity (nmol hr-1 mg-1)
0
25
50
75
100
125
150
175
200
NAE Concentration (Micromolar)

Figure 5. Inhibition of castor bean PLD α activity (expressed in *E. coli*) by increasing concentrations of NAE 12:0 (●), and NAE 14:0 (■). Protein (20 μg) was added to initiate the reaction. Assays were conducted as described in Figure 2. Data are from one experiment. Similar results were obtained in repeated experiments.

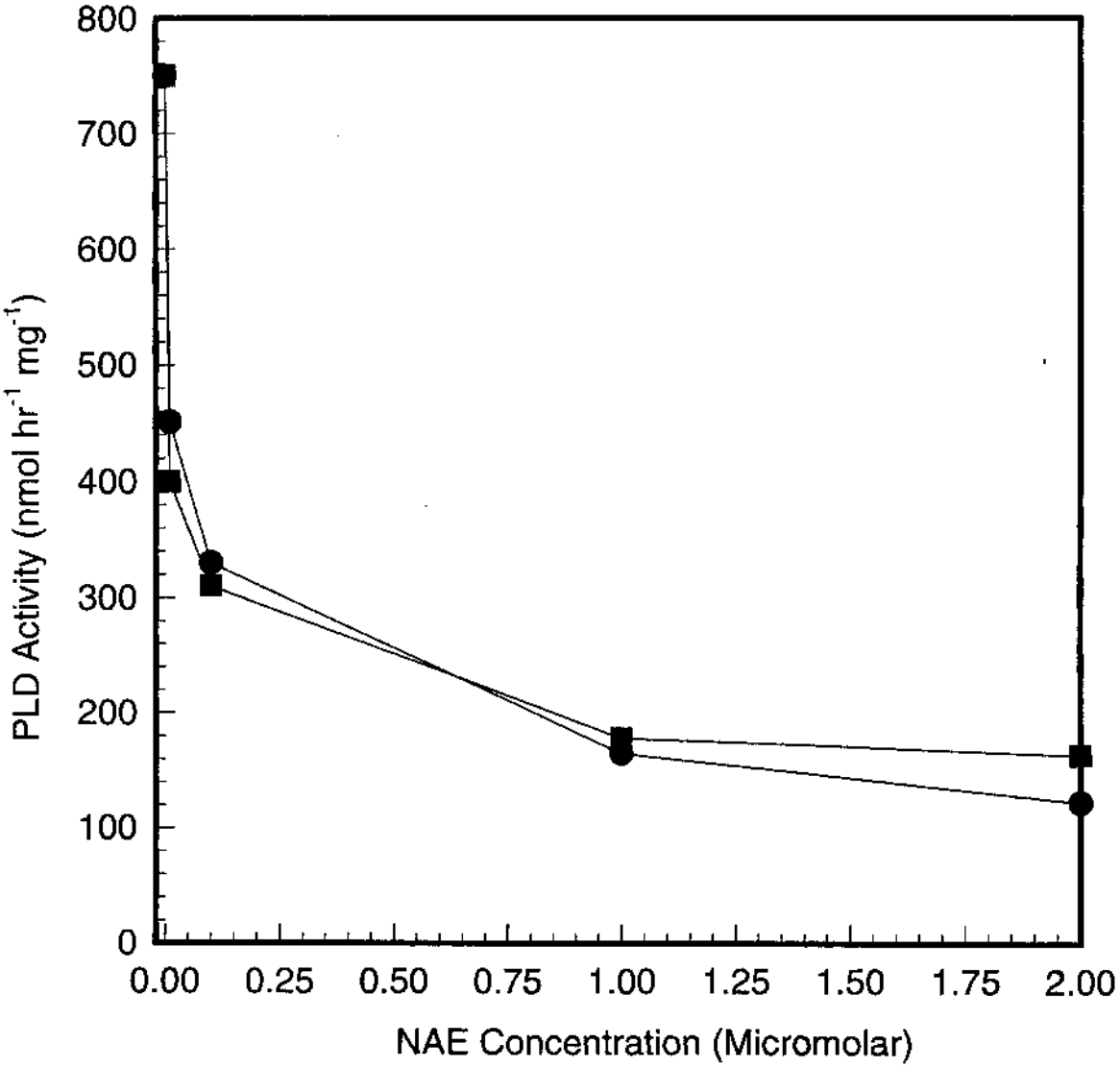

800
700
600
500
400
300
200
100
0
PLD Activity (nmol hr-1 mg-1)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
NAE Concentration (Micromolar)

Figure 6. Inhibition of commercially purified cabbage (Sigma catalog no. P0282) PLD α and *Streptomyces chromofuscus* (Sigma catalog no. P8023) PLD activity by increasing concentrations of NAE 12:0, and NAE 14:0. Protein (0.56 μg of cabbage PLD and 1.2 μg *Streptomyces chromofuscus* PLD) was added to initiate the reaction. (●) Cabbage PLD and NAE 12:0. (■) Cabbage PLD and NAE 14:0. (▲) *Streptomyces chromofuscus* PLD and NAE 12:0. (▼) *Streptomyces chromofuscus* PLD and NAE 14:0. Data are from one experiment. Similar results were obtained in repeated experiments.

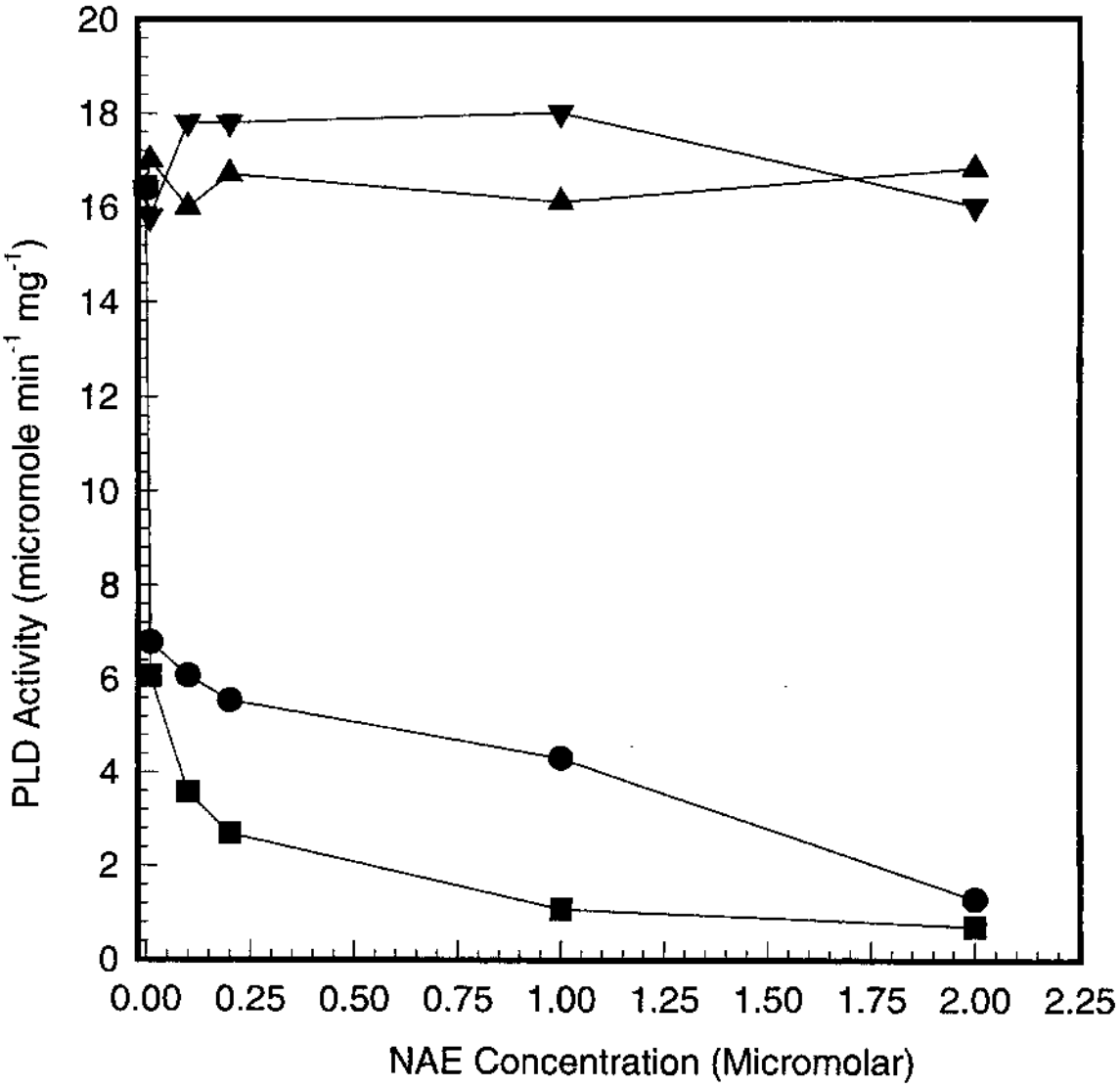

20
18
16
14
12
10
8
6
4
2
0
PLD Activity (micromole min-1 mg-1)
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
2.25
NAE Concentration (Micromolar)

Figure 7. Double reciprocal plot of initial velocity of cabbage PLD α versus substrate (PC) concentrations. PLD α activity was measured in the absence (●) and presence (■) of 0.01μM NAE 14:0. Analysis of the data by Michaelis-Menten kinetics indicated no change in the apparent Km (2.5mM) of the enzyme for PC (with or without NAE 14:0), while the apparent Vmax was reduced by 34% in the presence of NAE.

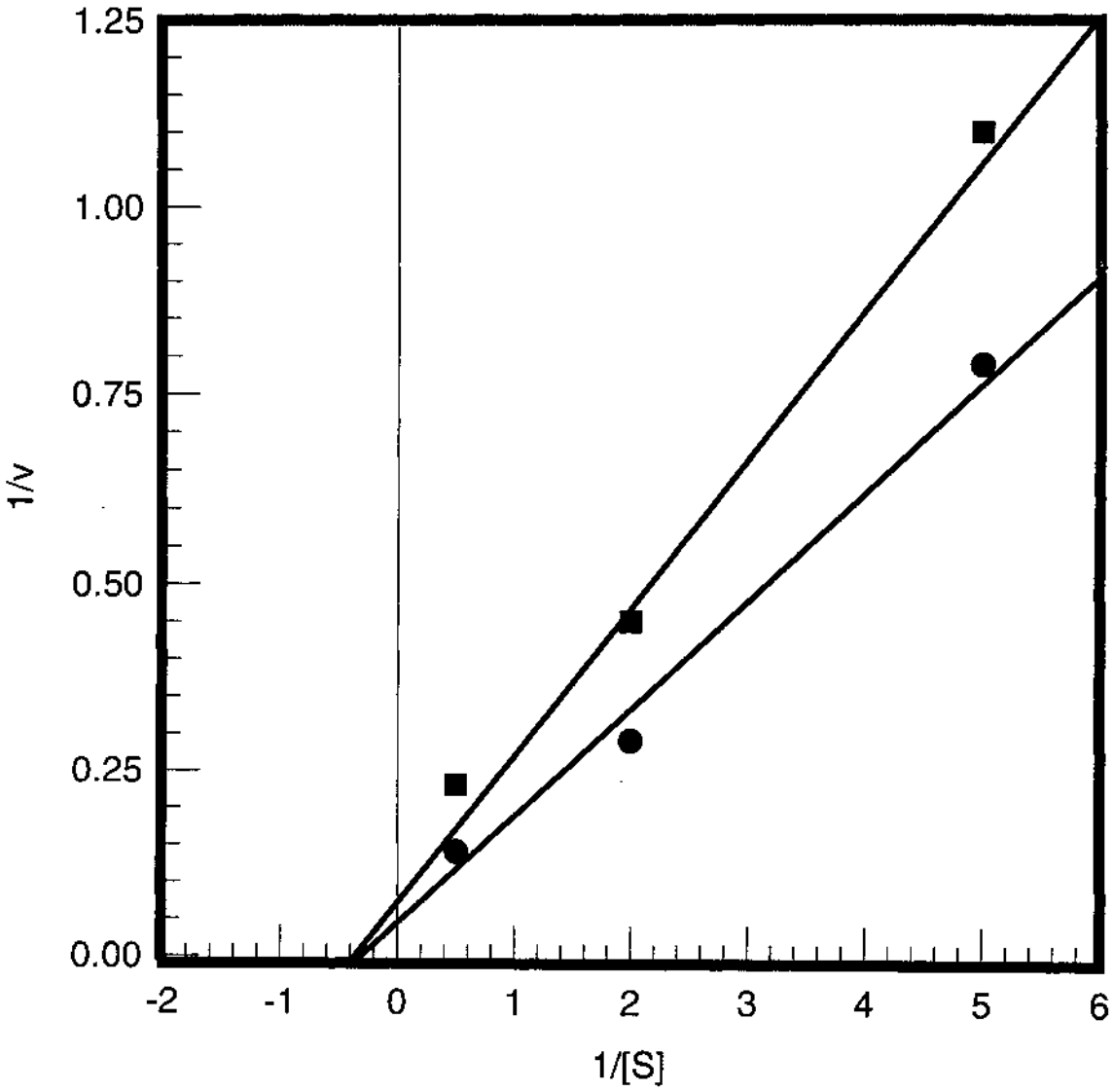

1.25
1.00
0.75
0.50
0.25
0.00
1/v
-2
-1
0
1
2
3
4
5
6
1/[S]

Table II. IC_{50} values of NAEs for the inhibition of castor bean PLD α expressed in *E. coli*. Values were estimated graphically from data in Figures 4 and 5.

N-acylethanolamines	IC_{50} values (μM)
12:0	0.13
14:0	0.10
16:0	5.00
18:0	10.00
18:1	40.00
18:2	30.00
18:3	80.00

Figure 8. Separation of PCR products subcloned into pZErO-2.1™ and digested with *Sac* I and *Xba* I. Lanes 2, 5, 8, and 11 are clones I3, I6, II1, and II6, respectively, not digested with any restriction enzyme. Lanes 3, 6, 9, and 12 are clones I3, I6, II1, and II6, respectively, digested with *Xba* I. Lanes 4, 7, 10, and 13 are of clones I3, I6, II1, and II6, respectively, digested with *Sac* I and *Xba* I. Size markers are located in lanes 1 and 14. Clone II1 and II6 contained inserts of ~1.2-kb and were chosen for sequencing analysis. DNA was separated in a 1.5% agarose gel, and visualized by staining with 0.I μg/ml ethidium bromide.

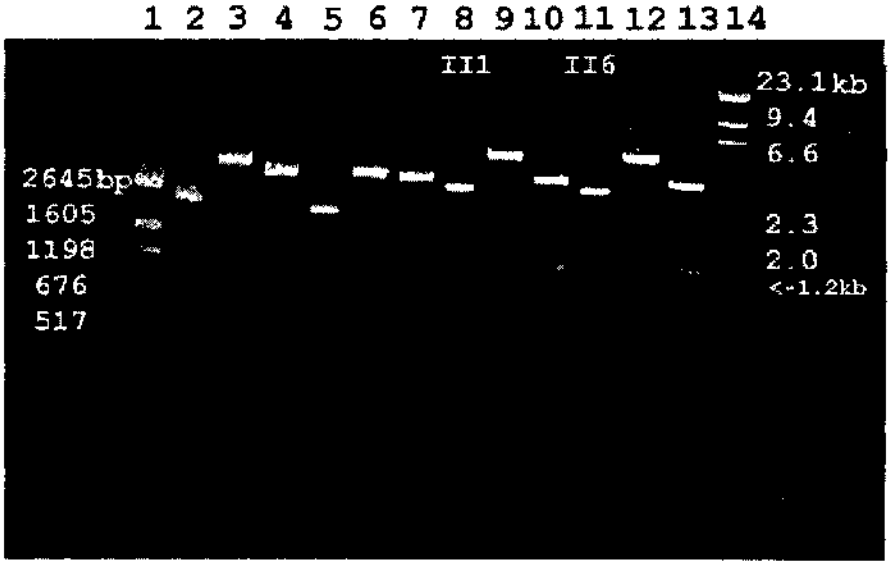

1 2 3 4 5 6 7 8 9 10 11 12 13 14
II1
II6
23.1kb
9.4
6.6
2.3
2.0
<-1.2kb
2645bp
1605
1198
676
517

Figure 9. Restriction analysis of clone II1. Single digestions were carried out with 2 µg of clone II1 plasmid DNA and 10 units of the following restriction enzymes: *Hin*d III (lane 2), *Kpn* I (lane 3), *Sac* I (lane 4), *Bam* HI (lane5), *Eco* RI (lane 6), and *Pst* I (lane 7). Lane 8 contains 2 µg of undigested clone II1 in pZErO-2.1™. DNA was separated in a 1.5% agarose gel, and visualized by staining in a 0.1 µg/ml ethidium bromide. Only the digestion with *Hin*d III revealed an internal restriction site (see 487-bp fragment in lane 2).

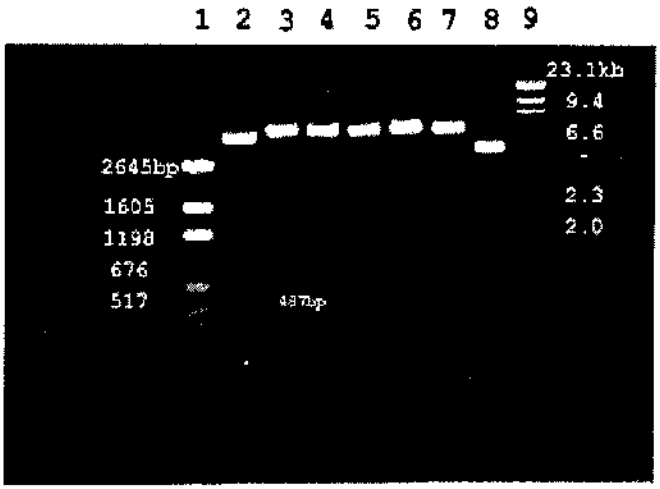

1 2 3 4 5 6 7 8 9
23.1kb
9.4
6.6
2.3
2.0
2645bp
1605
1198
676
517
487bp

Figure 10. Diagram of the multiple cloning site of pZErO-2.1™ vector with insert, clone II1. Restriction sites that flank the 1.2-kb insert, as well as the internal restriction site are designated. The position of the clone is with respect to the M13 forward and reverse primers as shown.

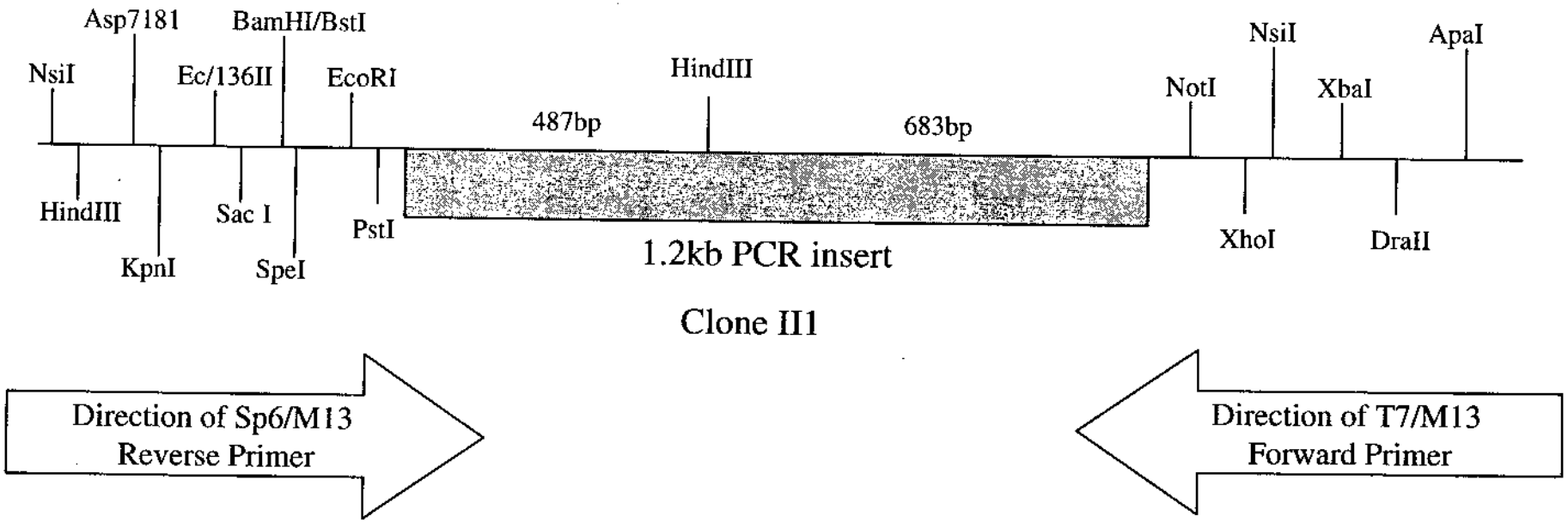
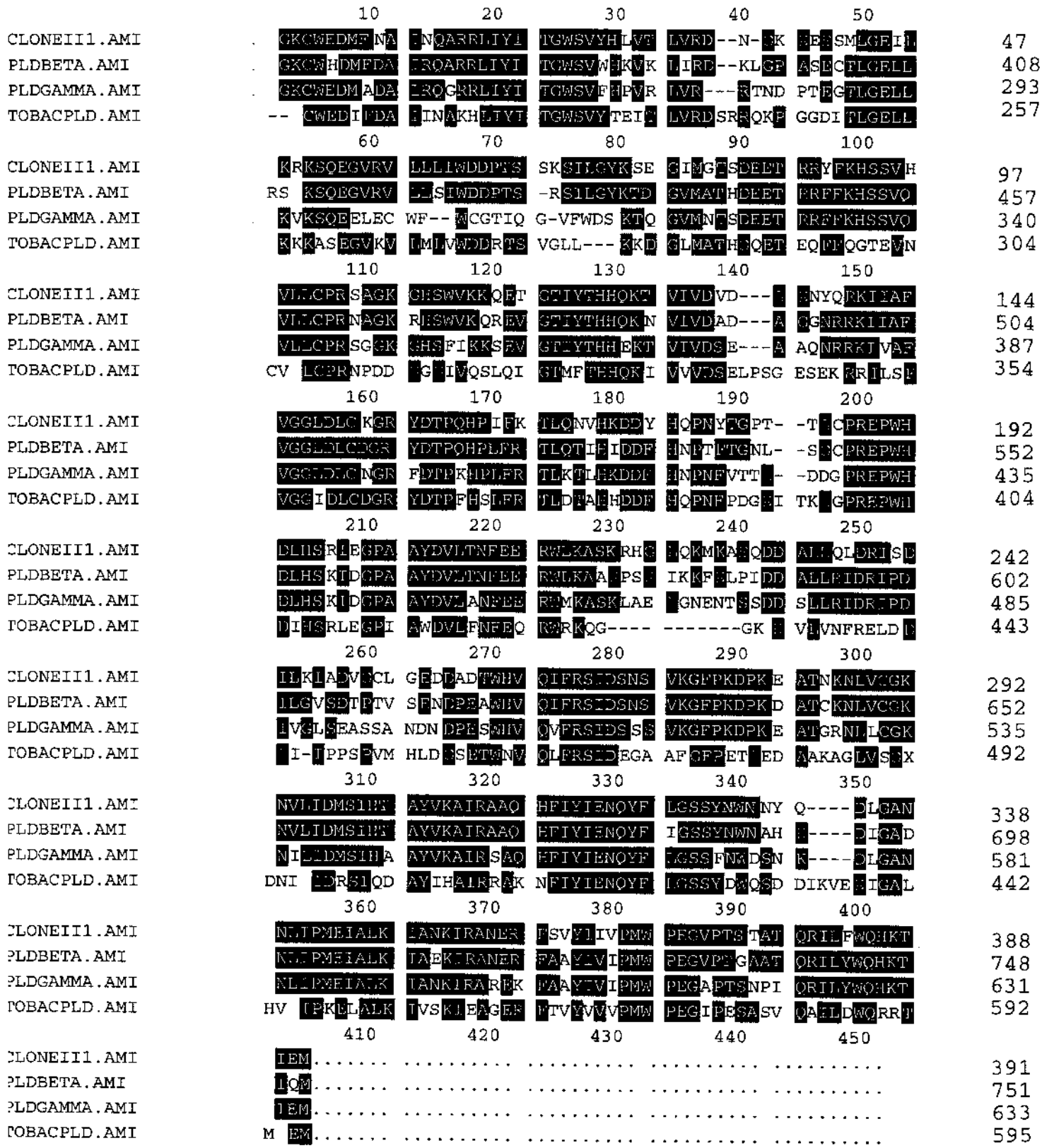

Asp7181
BamHI/BstI
NsiI
Ec/136II
EcoRI
HindIII
NotI
NsiI
XbaI
ApaI
487bp
683bp
HindIII
Sac I
PstI
KpnI
SpeI
XhoI
DraII
1.2kb PCR insert
Clone II1
Direction of Sp6/M13
Reverse Primer
Direction of T7/M13
Forward Primer

Figure 11. Nucleotide sequence and deduced amino acid sequence of clone II1. Clone II6 contained an identical sequence. Dash lines with arrows indicate position of forward (+5) and reverse (-1) PCR primers. The putative catalytic site is designated *HXKXXXXD*.

```
          9          18          27          36          45          54
5'  GGG AAG TGC TGG GAG GAC ATG TTC AAT GCA ATA AAT CAG GCT CGT CGG TTG ATT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gly Lys Cys Trp Glu Asp Met Phe Asn Ala Ile Asn Gln Ala Arg Arg Leu Ile
     ---------------------->
          63          72          81          90          99          108
    TAC ATT ACA GGA TGG TCA GTG TAC CAC CTA GTT ACA CTT GTT AGG GAT AAT GGA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Tyr Ile Thr Gly Trp Ser Val Tyr His Leu Val Thr Leu Val Arg Asp Asn Gly

          117         126         135         144         153         162
    AAA GCT GAG GAA AGC ATG TTA GGG GAA ATT CTC AAG AGG AAA TCC CAA GAA GGT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Lys Ala Glu Glu Ser Met Leu Gly Glu Ile Leu Lys Arg Lys Ser Gln Glu Gly

          171         180         189         198         207         216
    GTG AGA GTA CTG CTT CTC ATA TGG GAT GAT CCT ACC TCT TCG AAG AGC ATC TTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Arg Val Leu Leu Leu Ile Trp Asp Asp Pro Thr Ser Ser Lys Ser Ile Leu

          225         234         243         252         261         270
    GGA TAC AAA AGT GAA GGA ATC ATG GGA ACT AGT GAT GAA GAA ACT CGT CGC TAT
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gly Tyr Lys Ser Glu Gly Ile Met Gly Thr Ser Asp Glu Glu Thr Arg Arg Tyr

          279         288         297         306         315         324
    TTT AAG CAT TCT TCA GTG CAC GTG CTA CTT TGT CCC CGT TCT GCT GGA AAA GGG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Lys His Ser Ser Val His Val Leu Leu Cys Pro Arg Ser Ala Gly Lys Gly

          333         342         351         360         369         378
    CAC AGC TGG GTC AAA AAA CAG GAA ACT GGA ACA ATA TAC ACA CAT CAT CAG AAA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    His Ser Trp Val Lys Lys Gln Glu Thr Gly Thr Ile Tyr Thr His His Gln Lys
                                                            H   X   K
          387         396         405         414         423         432
    ACT GTA ATA GTG GAT GTG GAT GCT GGT AAT TAC CAG AGA AAG ATT ATC GCT TTC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Thr Val Ile Val Asp Val Asp Ala Gly Asn Tyr Gln Arg Lys Ile Ile Ala Phe
     X   X   X   X   D
          441         450         459         468         477         486
    GTT GGT GGC CTT GAT TTG TGC AAA GGG CGT TAT GAT ACT CCA CAA CAC CCT ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Val Gly Gly Leu Asp Leu Cys Lys Gly Arg Tyr Asp Thr Pro Gln His Pro Ile

          495         504         513         522         531         540
    TTT AAA ACA TTG CAA AAT GTG CAC AAA GAT GAC TAT CAT CAG CCT AAC TAC ACG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Phe Lys Thr Leu Gln Asn Val His Lys Asp Asp Tyr His Gln Pro Asn Tyr Thr

          549         558         567         576         585         594
    GGC CCT ACT ACC GGT TGT CCT AGA GAA CCT TGG CAT GAT TTA CAT AGT CGG ATC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gly Pro Thr Thr Gly Cys Pro Arg Glu Pro Trp His Asp Leu His Ser Arg Ile

          603         612         621         630         639         648
    GAG GGG CCT GCT GCA TAT GAT GTC CTA ACT AAC TTC GAG GAG CGC TGG TTG AAG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Glu Gly Pro Ala Ala Tyr Asp Val Leu Thr Asn Phe Glu Glu Arg Trp Leu Lys

          657         666         675         684         693         702
    GCT TCA AAG CGC CAT GGA CTT CAA AAG ATG AAA GCT TCA CAA GAT GAT GCA TTA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Ala Ser Lys Arg His Gly Leu Gln Lys Met Lys Ala Ser Gln Asp Asp Ala Leu

          711         720         729         738         747         756
    CTC CAA CTT GAC AGG ATT TCC GAC ATA TTA AAA ATA GCT GAT GTC CCT TGC CTA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Leu Gln Leu Asp Arg Ile Ser Asp Ile Leu Lys Ile Ala Asp Val Pro Cys Leu

          765         774         783         792         801         810
    GGA GAA GAT GAT GCA GAT ACG TGG CAC GTG CAG ATT TTC CGG TCG ATT GAC TCC
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Gly Glu Asp Asp Ala Asp Thr Trp His Val Gln Ile Phe Arg Ser Ile Asp Ser

          819         828         837         846         855         864
    AAC TCT GTT AAA GGT TTC CCC AAA GAT CCC AAA GAA GCC ACT AAC AAG AAT CTA
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
    Asn Ser Val Lys Gly Phe Pro Lys Asp Pro Lys Glu Ala Thr Asn Lys Asn Leu
```

```
        873         882         891         900         909         918
GTT TGT GGC AAG AAT GTG CTG ATA GAT ATG AGC ATA CAT ACT GCC TAT GTA AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Cys Gly Lys Asn Val Leu Ile Asp Met Ser Ile His Thr Ala Tyr Val Lys

        927         936         945         954         963         972
GCA ATC CGA GCT GCC CAA CAT TTC ATC TAC ATT GAG AAC CAG TAC TTC CTA GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Ile Arg Ala Ala Gln His Phe Ile Tyr Ile Glu Asn Gln Tyr Phe Leu Gly

        981         990         999        1008        1017        1026
TCC TCA TAC AAT TGG AAT AAC TAC CAA GAT TTA GGT GCA AAT AAC TTG ATA CCG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Ser Tyr Asn Trp Asn Asn Tyr Gln Asp Leu Gly Ala Asn Asn Leu Ile Pro

       1035        1044        1053        1062        1071        1080
ATG GAG ATT GCT CTA AAA ATT GCC AAC AAA ATA CGG GCA AAT GAG AGG TTT TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Met Glu Ile Ala Leu Lys Ile Ala Asn Lys Ile Arg Ala Asn Glu Arg Phe Ser

       1089        1098        1107        1116        1125        1134
GTA TAT ATA ATT GTT CCT ATG TGG CCA GAG GGT GTT CCA ACC AGT ACT GCT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Tyr Ile Ile Val Pro Met Trp Pro Glu Gly Val Pro Thr Ser Thr Ala Thr

       1143        1152        1161        1170
CAG AGA ATA CTT TTT TGG CAA CAC AAA ACC ATA GAG ATG 3'
--- --- --- --- --- --- --- --- --- --- --- --- ---
Gln Arg Ile Leu Phe Trp Gln His Lys Thr Ile Glu Met
                    <------------------------
```

Figure 12. Alignment of predicted amino acid sequence of clone II1 versus amino acid sequences of *Arabidopsis* PLD β (Accession No U84568), *Arabidopsis* PLD γ (Accession No. AF27408) and tobacco PLD α (Accession No. Z84822). Alignments were made with DNASIS software (HIBIO DNASIS for Windows, version 2). Identical amino acid residues are indicated in black.


10 20 30 40 50
CLONEII1.AMI 47
PLDBETA.AMI 408
PLDGAMMA.AMI 293
TOBACPLD.AMI 257
60 70 80 90 100
CLONEII1.AMI 97
PLDBETA.AMI 457
PLDGAMMA.AMI 340
TOBACPLD.AMI 304
110 120 130 140 150
CLONEII1.AMI 144
PLDBETA.AMI 504
PLDGAMMA.AMI 387
TOBACPLD.AMI 354
160 170 180 190 200
CLONEII1.AMI 192
PLDBETA.AMI 552
PLDGAMMA.AMI 435
TOBACPLD.AMI 404
210 220 230 240 250
CLONEII1.AMI 242
PLDBETA.AMI 602
PLDGAMMA.AMI 485
TOBACPLD.AMI 443
260 270 280 290 300
CLONEII1.AMI 292
PLDBETA.AMI 652
PLDGAMMA.AMI 535
TOBACPLD.AMI 492
310 320 330 340 350
CLONEII1.AMI 338
PLDBETA.AMI 698
PLDGAMMA.AMI 581
TOBACPLD.AMI 442
360 370 380 390 400
CLONEII1.AMI 388
PLDBETA.AMI 748
PLDGAMMA.AMI 631
TOBACPLD.AMI 592
410 420 430 440 450
CLONEII1.AMI 391
PLDBETA.AMI 751
PLDGAMMA.AMI 633
TOBACPLD.AMI 595

CHAPTER IV

DISCUSSION

This study demonstrates, for the first time, that NAPE is not uniformly hydrolyzed by all known plant PLD isoforms. In fact, the hydrolysis of NAPE only occurred with two distinct forms of PLD, PLD β and γ. In addition, evidence collected also indicates the possibility of NAE regulation of PLD α *in vivo*. Finally, a tobacco cDNA fragment was isolated that contains a PLD characteristic HXKXXXXD catalytic motif and has high homology with the *Arabidopsis* PLD β, indicating that tobacco cells, which produce NAE express PLD β.

Biochemical Regulation of PLD

PLD isoforms were expressed in *E. coli* to examine the activity toward NAPE. The ability of PLD β and γ to hydrolyze NAPE marks a key difference from PLD α. PLD δ hydrolysis has not yet been shown (Wang et al., 1998), but it was tested in these studies using conditions as described for calcium-dependent assays as well as those conditions described for PIP_2-dependent assays. Most likely the appropriate environment/activator has yet to be used to activate this isoform of PLD. Activity of the PLD β and γ isoforms was previously demonstrated in membrane fractions (Pappan et al., 1998). In addition, PLD activity toward NAPE was reported in microsomal fractions of

tobacco cells (Chapman et al., 1998). This activity may be attributed to that of the PLD β or γ isoforms.

PLD α, β, and γ showed hydrolytic activity towards PC, PE, and PG, but PS and NAPE only served as substrates to PLD β and γ. The hydrolysis of these five phospholipids by PLD β and γ occurred under substantially different conditions than that used for PLD α activity. PLD β and γ required PIP_2 for activity towards all five of the phospholipids tested. In addition to PIP_2, PE was required for hydrolysis of NAPE, PG, and PC by PLD β and γ.

Recent studies have established that PIP_2 is required for hydrolysis of PC by PLD β and γ (Qin et al., 1997; Pappan et al., 1997b). Isoforms of PLD in mammals and yeast also showed a requirement of PIP_2 for PLD activity (Kodaki et al., 1997; Hammond et al., 1995; Waksman et al., 1996). Recent studies showed plant PLD binding affinity for PIP_2 using glutathione-S-transferase fusion protein constructs, in which radiolabeled PIP_2 bound to GST-PLD β greater than GST-PLD γ (Qin et al., 1997). The requirement for PIP_2 for PLD activity also wassupported by the identification of putative PIP_2 binding domains identified in the amino acid sequences of PLD β and γ (Qin et al., 1997).

The amount of PIP_2 needed for optimal activity in plants was around 8 mol%, but was active with as little as 1 mol% when tested in lipid vesicles (Pappan et al., 1997b). The amount of PIP_2 estimated is 0.05% of the total phospholipid in plants. Thus, this raises the question if activating levels are present in plant membranes. This question was recently addressed by Pappan and Wang by (Pappan and Wang, 1998) suggesting the possibility of PIP_2 concentration being sufficient due to its asymmetric distribution, with

greater amounts occurring on the inner leaflet of the plasma membrane. There is also evidence reported for mammalian cells, that PIP_2 is concentrated in the caveolae which are small, plasma membrane invaginations that have been suggested to play a role in cell signaling (Pike and Casey, 1996). Also, PIP was able to activate the PLDs, and perhaps the combination of PIP and PIP_2 could influence the activity of the PLD in an *in vivo* environment (Pappan and Wang, 1998). Another possible way of activation may be through the initial hydrolysis of phospholipids producing PA, which then stimulates the synthesis of PIP_2 from PIP by PI-4-phosphate kinase (Qin et al., 1997).

In addition to PIP_2, PLD β and γ require lipid vesicles predominately made of PE for the hydrolysis of NAPE. In animals, bovine kidney PLD required PE in mixed vesicles for activity (Nakamura et al., 1996). The requirement of a relatively high concentration of PE (50 mol% or more) for PLD β and γ activities toward NAPE indicates that PE is not acting as a cofactor for PLD β and γ, but rather, it affects the vesicular conformation (Pappan et al., 1998). Mixed phospholipid vesicles with a substantial amount of PE form inverted hexagonal phases (Lafleur et al., 1990; Cullis et al., 1986). This may mean that PLD β and γ are relatively inactive in a regular bilayer and their substrates are presented in lipidic particles (Pappan et al., 1998). Nonlamellar phases are reported to occur during membrane budding and fusion of two bilayers (Cullis et al., 1986). In addition, mammalian PLD has been proposed to be involved in vesicular trafficking and membrane fusion (Hammond et al., 1995).

The formation of inverted hexagonal phases may not be the only property that activates PLD β and γ. In plant cells, the biological membranes are composed primarily

of PC rather than PE, although an asymmetry exists with the majority of PE located on the interior of membrane bilayers. This location of PE on the interior of membranes may be necessary for membrane-protein associations to occur at physiological levels of calcium as indicated by a study which found four cytoplasmic proteins bound in a calcium dependent manner to membranes that contained PE rather than PC (Bazzi et al., 1992).

The requirement of both PIP_2 and a high concentration of PE for NAPE hydrolysis by PLD β and γ indicates that the two PLDs are highly regulated by membrane conformation and composition. The ability of only PLD β and γ to hydrolyze NAPE in a different membrane lipid environment than that of PLD α may underlie a mechanism that differentially activates the PLDs in the cell.

The concentration of calcium needed for PLD β and γ activity is also substantially different than that of PLD α. PLD β and γ require micromolar concentration of calcium for maximal activity; whereas, PLD α requires millimolar concentration of calcium for activity (Pappan et al, 1997a). Calcium concentration differences may be due to differences within the calcium-phospholipid binding (C2) domain. PLD α has a loss or substitution of three of the conserved acidic amino acids in exchange for neutral or positively charged amino acids; hence, this may be imparting a loss of calcium sensitivity (Kopka, et al., 1998).

The hydrolysis of NAPE by PLD β and γ leads to the formation of NAE. In this study, I identify NAE, a naturally occurring lipid, as a potent inhibitor of plant PLD α. This lipid has been implicated previously in several mechanisms in mammalian tissues

including cell signaling as an endogenous ligand for the cannabinoid receptor (Schmid et al., 1996; Beltramo et al., 1997). Most recently, NAE 12:0 and 14:0 were found to accumulate in the culture medium of elicitor-treated tobacco cells. In addition, a microsomal phospholipase D activity was discovered that hydrolyzed NAPE to form NAE (Chapman et al., 1998).

Several inhibitors of mammalian phospholipase D have been identified. These inhibitors include fodrin (Lukowski et al., 1996), synaptojanin (Kim et al., 1996), and clathrin assembly protein (Lee et al., 1997) as well as some lipids. An oleate-dependent PLD from rat brain was inhibited by several acidic phospholipids, of which PIP_2 was the most effective inhibitor (Kanfer et al., 1996). In contrast to this was the PIP_2-stimulated PLD, which was inhibited by oleate (Hammond et al., 1995). This unique interaction in which an activator for one PLD isoform is an inhibitor of another gives an example of possible PLD regulation and "crosstalk" between different PLD isozymes. The hydrolysis of NAPE by PLD β and γ to form NAE and its inhibition of PLD α may be yet another form of regulation between the different PLD isozymes.

Other lipid inhibitors of PLD in mammalian systems include ceramide (Venable et al., 1996), alkylphosphate esters (Dittrich et al., 1996) and lysophosphatidylserine (Kawabe et al., 1998). Ceramide, a sphingolipid, was also shown to be an inhibitor of PLD. This inhibition is a result of ceramide interacting with protein kinase C-mediated activation of PLD (Venable et al., 1996). Lysophosphatidylserine is an effective inhibitor of both oleate-dependent PLD, ARF-dependent PLD, and PIP_2-dependent PLD (Kawabe et al., 1998). The inhibition of plant PLD by lysophosphatidylethanolamine

(LPE) recently has been shown to occur. LPE is a lipid-derived senescence retardant of leaves, flowers, and postharvest fruits (Ryu et al., 1997). NAE inhibition of PLD α is only the second lipid inhibitor of plant PLDα. This evidence along with the release of NAE in response to fungal elicitor provides increasing evidence that NAE may function as a signal molecule in plants.

N-Acylethanolamines (NAE) with different acyl chains were examined for their relative effectiveness on PLD α activity. *N*-acylethanolamine 12:0 and NAE 14:0 showed the most effective inhibition of plant PLD α. The results of this study is in contrast to other studies where acyl chain length was examined as a factor affecting the inhibition of PLD. Lysophosphatidylethanolamine (Ryu et al., 1997) and akylphosphate esters (Dittrich et al., 1996) both were shown to increase inhibition of PLD with increasing chain length. Lysophosphatidylethanolamine 18:1 was the most potent inhibitor of both castor bean and cabbage PLD α. The ability of LPE 18:1 to effectively inhibit PLD was in a concentration range of 40 μM to 200 μM (Ryu et al., 1997). Lysophosphatidylserine inhibition of mammalian PLD ranged from 1 μM to 10 μM (Kawabe et al., 1998). My results show potent inhibition of PLD α activity from 0.1 μM to 1μM for NAE 12:0 and NAE 14:0. These results are significant for two reasons. First, the molecular species that is the most potent inhibitor is also the molecular species that is released from tobacco cells upon treatment with fungal elicitor. Second, the low concentrations needed for inhibition of plant PLD α makes it plausible to consider NAE as a possible lipid mediator *in vivo*..

Inhibition of castor bean PLD α by NAE 14:0 is believed to be through noncompetitive binding based on enzyme-kinetic analysis. Noncompetitive inhibition of PLD α by LPE was also reported (Ryu et al., 1997). The apparent Km determined during my studies was comparable to other published apparent Km values for castor bean and cabbage PLD α. The apparent Vmax in the absence of inhibitor is also comparable to other published apparent Vmax values(Ryu et al., 1997; Wang et al., 1993). In addition to examining the type of inhibition PLD by NAE 14:0, an apparent K_I was calculated. The apparent K_I for NAE 14:0 was 0.02 μM, which is consistent with the IC50 value of 0.03 μM. This is in contrast to the IC_{50} value for castor bean PLD α of 0.1 μM for NAE 14:0. The discrepancy in the values could be due to the presence of interfering compounds in the *E. coli* lysate (not present in the purified cabbage PLD preparation), which may bind to NAE making it inaccessible to the PLD.

The mechanism of PLD α inhibition by NAE is not fully understood. NAE may be interacting directly with the enzyme to change its native conformation and decrease its activity. Another explanation is the possibility of NAE interacting with the lipid environment to influence the presentation or accessibility of substrate. Since NAE readily incorporates into the lipid surface, the possibility of diluting the effective concentration of the substrate must also be considered (Carman et al., 1995). This is most readily ruled out as a contributing factor due to the extremely high ratio of substrate to inhibitor concentration.

Efforts are now being directed toward determining the role of NAE in plant pathogen interaction. Preliminary results suggest that NAE is involved in the inhibition

of the well-characterized, elicitor-induced H^+/K^+ exchange response (Tripathy et al., 1997), and NAE may be involved in the activation of defense response genes, phenylalanine ammonia lyase (PAL). The evidence presented in this study supports the hypothesis that NAE is a biologically active molecule.

Molecular Heterogeneity of PLD

In the present study, I report the isolation of a tobacco cDNA fragment, which contains an ORF amino acid sequence highly homologous to a portion of the phospholipase D β from *Arabidopsis thalania*. The tobacco cDNA fragment has 74%, 65% and 52% identity to *Arabidopsis* PLD β, *Arabidopsis* PLD γ, and tobacco PLD α, respectively.

Phospholipase D sequences from maize, rice, castor bean, tobacco, black-eyed pea and rapeseed all share a high degree of homology to the *Arabidopsis* PLD α. *Arabidopsis* PLD α and castor bean PLD are 80% identical, and the PLD α amino acid sequences of monocotyledons are approximately 90% identical. In contrast the *Arabidopsis* PLD β and γ sequences only share 45-50% identity to PLD α(Wang, 1997). Phylogenetic analysis indicates PLD β and γ are evolutionarily divergent from PLD α, and PLD β and γ are more closely related to the proteins cloned from yeast and human than to PLD α (Pappan et al., 1997b). *Arabidopsis* PLD β and γ, along with human and yeast PLDs, have basic pI values ranging from 7.6-9.3. PIP_2 is not only a requirement for PLD β and γ activation, but also for activation of isoforms of PLD in humans and yeast. The isoelectric point for the PLD α forms in plants are acidic with a range of 5-6.

Comparison of the isoelectric points and catalytic properties of the PLDs support the phylogenetic groupings. In addition to these differences, it was demonstrated by Southern blotting analysis that *Arabidopsis* PLD α, β, and γ were encoded by distinct genes (Pappan et al., 1997a). The isolation of a putative tobacco PLD β fragment aids in establishing the presence of phospholipase D β in plants.

Isolation of full length cDNAs for the tobacco PLD β isoform, will facilitate future functional studies of the role of this enzyme *in vivo*. Future research toward the manipulation of PLD β levels in transgenic plants using this cDNA should shed light on the role of PLD β in plants. Furthermore, the altering of the expression of PLD β will aid in elucidating the physiological role of NAE in plants. Additional biochemical characterization examining the interaction of NAE on PLD α using crosslinking agents will determine if a binding sit exists on PLD α for NAE.

Summary

Together these data suggest several novel concepts regarding NAPE hydrolysis and NAE's possible role in regulation of PLD α. In addition, the isolation of a cDNA fragment from tobacco with high homology to the *Arabidopsis* PLD β contributes to establishing PLD isoforms in higher plants.

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