Effect of Processing Conditions on Phospholipase D Activity of Corn Kernel Subcellular Fractions

Gopinadhan Paliyath,*,† Reena G. Pinhero,† Rickey Y. Yada,† and Dennis P. Murr‡

Department of Food Science and Division of Horticulture, Department of Plant Agriculture, University of Guelph, Guelph, Ontario N1G 2W1, Canada

The influence of physicochemical conditions on the phospholipase D (PLD) activity of subcellular preparations of sweet corn (*Zea mays* L. cv. Peaches and Cream) kernels has been studied. The microsomal, mitochondrial, and cytosolic preparations of corn kernels possessed PLD activity albeit at varying proportions. The microsomal and cytosolic PLD activities were stimulated 2-fold between 5 and 15 °C. Ethanol had varying modulatory effects on PLD activity. By contrast, acetaldehyde was a potent inhibitor of PLD. As well, a naturally occurring C_6 aldehyde such as hexanal and an alcohol such as hexanol inhibited PLD activity efficiently. Divalent cations such as calcium chloride and magnesium chloride stimulated PLD activity at micromolar levels. Monovalent cations such as KCl and NaCl did not appear to affect PLD activity. Partial purification of PLD from the microsomal, mitochondrial, and cytosolic fractions separately revealed four major isoforms with relative molecular masses of 200, 140−150, 102−108, and 60−66 kDa. The importance of PLD in the maintenance of processed food quality is discussed.

Keywords: *Chilling; membrane; phospholipase D; processing; sweet corn kernels*

INTRODUCTION

Maturation and ripening of several kinds of perishable plant produce are associated with the catabolic breakdown of cellular structures such as the membrane and the cell wall, which is a normal process in the development of ideal organoleptic quality. Untimely destruction of cellular integrity of the produce, as occurs during processing (cutting, wounding, blending, maceration, etc.) or storage (injury due to chilling) can lead to accelerated destruction of cellular structures, sometimes resulting in the loss of quality of the intended product. Preservation of membrane compartmentation is a must for maintaining the quality of unprocessed perishable plant produce such as corn kernels, leafy vegetables, flowers (cauliflower, broccoli), and fruits. Many of these commodities are used for fresh consumption as well as processing, such as canning, making of soups, jams, and sauces, and blending for juice-making. During processing, various produce items are subjected to conditions such as chilling, freezing, heating, and mixing with salts, preservatives, and solvents, which destroy the cellular compartmentation. An excess of these operations can lead to the loss of processed food quality as observed in the development of mushiness and off flavors of several processed products.

Phospholipase D (PLD) is a ubiquitous and key enzyme that catalyzes the hydrolysis of membrane phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylglycerol to yield phosphatidic acid and the respective headgroup (Kates, 1955; Galliard, 1980; Exton, 1997). In vitro, PLD also catalyzes the exchange of phospholipid headgroups with primary alcohols such as methanol and ethanol, leading to the formation of phosphatidyl alcohol, a reaction called transphosphatidylation (Galliard, 1980; Cockfort, 1997). PLD is widespread in various plant species (Quarles and Dawson, 1969; Galliard, 1980). Soluble and membrane-associated PLDs have been reported in various plant species (Yoshida, 1979; Galliard, 1980; Xu et al., 1996). Various physiological processes such as seed germination (Wang et al., 1993), growth of seedlings (Herman and Chrispeels, 1980), stress-induced changes, and senescence (Yoshida, 1979; Paliyath and Droillard, 1992) have been suggested to be regulated by PLD. A high activity of PLD has been found in storage tissues, especially in seeds (Heller, 1978). Activation of PLD is elicited by a variety of agonists in different cell types, leading to the tandem generation of messengers, namely phosphatidic acid and diacylglycerol, that affect many significant cellular processes (Paliyath and Droillard, 1992; Exton, 1997).

Previous studies have shown that exposure of black locust bark tissues to frost as well as wounding (Yoshida, 1979) and chilling of maize seedlings (Pinhero et al., 1998) resulted in an increase in PLD activity, causing very significant lipid degradation and membrane deterioration. It has been reported that PLD is responsible for the hydrolysis of spherosome membrane phospholipids, which causes triacylglycerol leakage out of spherosomes and its subsequent degradation (Takano et al., 1989). Increased association of PLD with microsomal membranes has been proposed to promote PLD-mediated degradation of membrane lipids during γ-irradiation and senescence (Voisine et al., 1993; Ryu and Wang, 1995). During tomato fruit ripening, decreased fluidity of microsomal membrane has been reported to activate PLD and increase membrane phospholipid catabolism (McCormac et al., 1993). Under such condi-

* Author to whom correspondence should be addressed [telephone (519) 824-4120, ext. 4856; fax (519) 824-6631; e-mail gpaliyat@evbhort.uoguelph.ca].

† Department of Food Science.

‡ Division of Horticulture.

10.1021/jf981048n CCC: $18.00
Published on Web 06/11/1999

tions, PLD activity proceeds in an autocatalytic fashion leading to the total destruction of the structural and functional organization of the membrane and abolition of membrane compartmentation (Paliyath and Droillard, 1992).

Sweet corn is widely used as a vegetable for fresh consumption and for manufacturing other processed food products. Processing and preservation of the product involves mechanical as well as cold treatments that can activate PLD and degrade the lipids, resulting in poor quality of the kernels. In this study, we have investigated the regulation of PLD in kernels of sweet corn by various physicochemical conditions to define optimal conditions and methods of storage and processing for minimizing membrane damage and preserving quality. As well, an attempt has been made to partially purify PLD from membrane fraction and the cytosol separately to understand the relative distribution of PLD in the cell.

MATERIALS AND METHODS

Protein Extraction. Sweet corn (*Zea mays* L. cv. Peaches and Cream) was used for this study. The kernels were obtained locally from a grocery store and stored frozen at −20 °C until they were used for protein extraction. Frozen kernels were homogenized in 0.1 M Tris-HCl buffer at pH 7.5 essentially as described by Pinhero et al. (1998), except that sucrose was not included in the homogenizing buffer. The mitochondrial, microsomal, and cytosolic fractions were collected by differential centrifugation (Pinhero et al., 1998). The fraction comprising the starch granules was removed by centrifuging the samples at 2500*g* for 10 min. The resulting supernatant was subjected to centrifugation at 15000*g* to pellet the mitochondrial membrane fraction (Edwards and Gardestrom, 1987). The supernatant from this step was centrifuged at 105000*g* to pellet the microsomal membranes. The supernatant comprising the cytosol was subjected to ammonium sulfate fractionation at 30 and 60% saturation. The precipitated protein was collected by centrifugation at 15000*g* and redissolved in 10 mM Tris-HCl (pH 7.0) and dialyzed overnight with the same buffer, also containing 0.1 mM DTT, with two changes of 2 L each. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as a standard. All steps of protein extraction procedure were carried out at 4 °C.

Enzyme Assay. PLD activity was determined as described by Pinhero et al. (1998) by measuring the release of radiolabeled choline from 1,2-dipalmitoyl-L_3-phosphatidyl(*N*-methyl-^{3}H)choline at 23 °C. The basic assay mixture contained 0.1 M Tris-HCl (pH 7.5), 0.2 mM EGTA, membrane or cytosol protein (2.5 μg), and ∼100000 dpm of choline-labeled phosphatidylcholine in 0.1% (v/v) Triton-X 100 (0.01% final), to make a total volume of 1 mL. To study the regulation of PLD by alcohols, aldehydes, and metal ions, an appropriate amount of the respective reagent solution was added to the basic reaction mixture. Similarly, citrate and Tris-HCl buffers were used to study the effect of pH on PLD activity. The reaction was terminated after 10 min by adding 100 μL of 4 N HCl followed by 1 mL of chloroform/methanol (2:1, v/v) and left overnight. The amount of [^{3}H]choline released during the reaction was determined by mixing a 0.5 mL aliquot of the aqueous phase directly into 5 mL of scintillation fluid (Ecolume, ICN) and determining the amount of radiolabel using a Beckman LS 6800 Scintillation counter (Beckman Instruments Inc.).

Purification of PLD. Mitochondrial, microsomal, and cytosolic fractions from several extractions were pooled and stored frozen at −80 °C. The cytosolic fraction was clarified by filtration through Whatman 935 AH glass fiber filters and subjected to ammonium sulfate fractionation at 30% saturation. The pellet obtained from the centrifugation of the precipitated protein was dissolved and dialyzed against 10 mM Tris-HCl containing 0.1 mM DTT and 0.1 mM EDTA for 20 h, with three changes of the buffers (2 L each). The protein solution was clarified by centrifugation (15000*g*) and loaded on a DEAE-Sephacel column (50 cm × 2.5 cm) equilibrated with 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM DTT, at the rate of 0.3 mL/ min. The column was washed at 30 mL/h with the same buffer until no UV-absorbing material was detectable in the effluent. Bound protein was eluted by a NaCl gradient of 0−1 M NaCl in the wash buffer. Fractions of 7 mL were collected at a flow rate of 1 mL/ min and assayed for protein concentration, PLD activity, and absorbance at 280 nm. Fractions containing peak levels of enzyme activity were pooled separately into two major fractions and precipitated with the addition of ammonium sulfate at 90% saturation. The pellet was collected after centrifugation at 15000*g* for 15 min and dissolved in 10 mM Tris-HCl containing 0.1 mM DTT. The protein was dialyzed against 2 L of 10 mM Tris-HCl (pH 7.0) containing 0.1 mM EDTA and 0.1 mM EGTA and concentrated by lyophilization. The dry powder was stored frozen at −80 °C.

For purification of mitochondrial and microsomal PLD, the membrane protein was solubilized by the addition of 0.5% (v/v) Triton X-100. Unsolubilized materials were separated by centrifugation at 105000*g* for 1 h. The clear supernatant was subjected to ion exchange chromatography on DEAE-Sephacel as described earlier. Fractions containing peak levels of PLD activity were pooled and concentrated by lyophilization. The dry powder was stored frozen at −80 °C. Further purification was achieved by gel filtration on a Sephacryl S-300 HR column (50 cm × 1 cm) using a Waters 616/626 FPLC system (Waters Canada). The column was equilibrated with 10 mM Tris-HCl (pH 7.0). The protein sample equivalent to 100 μg of protein was fractionated on the column at a flow rate of 0.5 mL/min using10 mM Tris-HCl (pH 7.5) containing 0.02 mM EDTA and 0.02 mM DTT. Fractions of 0.5 mL were collected and assayed for PLD activity. The relative molecular masses of the major peaks eluted were calculated using a standard calibration curve prepared with gel filtration molecular mass markers (MW-GF-200 kit, gel filtration molecular markers: cytochrome *c*, 12.4 kDa; carbonic anhydrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; and β-amylase, 200 kDa) obtained from Sigma Chemical Co., St. Louis, MO.

Chemicals. Phosphatidylcholine [1,2-dipalmitoyl-L_3-phosphatidyl(*N*-methyl-^{3}H)choline (3.00 TBq mmol^{-1}] was purchased from Amersham Life Sciences. DEAE-Sephacel, Sephacryl S-300, and other chemicals were purchased from Sigma Chemical Co.

RESULTS

The existence of several isoforms of PLD has recently been confirmed in plants such as soybean and castor bean, the synthesis of which is temporally regulated during development (Dyer et al., 1994; Ryu and Wang, 1995; Ryu et al., 1996). PLD exists as membranous and cytosolic forms. In the previous studies, PLD was isolated from the total tissue homogenate and potential differences in biochemical properties of the cytosolic and membranous forms have not been given due consideration. Moreover, the properties of PLD in chloroplast, mitochondria, endoplasmic reticulum, plasma membrane, and vacuole have not been studied. In a food system such as corn kernels, the properties of these compartmentalized PLD could be modulated differently under processing conditions. Therefore, properties of PLD were studied in the mitochondrial and plastid fractions (15000*g* pellet), the microsomal fraction that comprises endoplasmic reticulum, plasma membrane, and vacuolar membranes, and the cytosolic fraction separately.

Estimation of PLD activity by monitoring the liberation of choline is a convenient and rapid method and is

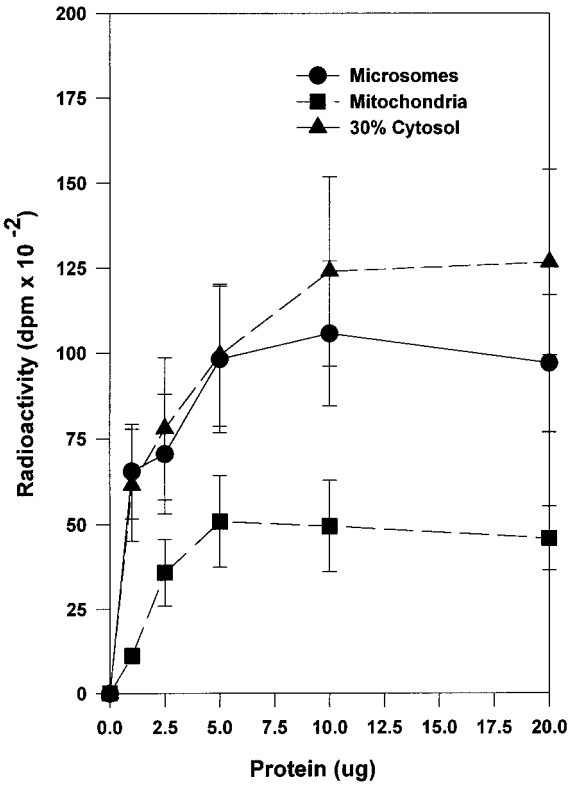

Figure 1. Effect of protein on the activity of PLD in subcellular preparations of sweet corn kernel. Membrane and soluble fractions were extracted from sweet corn kernel as described under Materials and Methods. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine [1,2-dipalmitoyl-L_3-phosphatidyl(*N*-methyl-^{3}H)choline] in a 1-mL reaction mixture during an incubation period of 10 min. The values are mean ± SE from four separate experiments.

comparable to other methods of estimating PLD such as with the use of fluorescent substrates or the estimation of phosphatidylethanol formed (Harris et al., 1995; Pinhero et al., 1998). This method has been consistently used in our laboratory for several studies (Paliyath and Thompson, 1987; Todd et al., 1992; Pinhero et al., 1998).

Effect of Protein on PLD Activity. PLD activity showed a linear increase in activity with increasing protein levels in the assay mixture and attained a plateau after the 10 μg protein level in the cytosolic, microsomal, and mitochondrial fractions (Figure 1). Ammonium sulfate fractionation of the cytosolic fraction at 30 and 60% saturation showed the presence of PLD activity in both, albeit at different specific activities (data not shown). The protein pellet that resulted after 30% saturation appeared to possess higher specific and total activities and was used in all further experiments. However, cytosolic PLD activity varied considerably and possessed lower specific activity than that in the microsomes. The specific activity of PLD in the mitochondrial fraction was the lowest and appeared to be nearly half of the specific activity of microsomal PLD. On a specific activity basis, 30% cytosol and the microsomal fraction possessed PLD activity that liberated choline at a rate of 3×10^6 dpm/mg of protein/min. The specific activity of the mitochondrial fraction was nearly half of that in the cytosol and the microsomal fractions and reached 1.48×10^6 dpm/mg of protein/min. The total activity of PLD was the highest in the cytosol followed by the mitochondrial and the microsomal fractions, the activities being 179×10^4, 81.7×10^4, and 38.5×10^4 dpm of choline released/g of fresh weight. PLD activity was rapid, and the liberation of choline reached a plateau within 2.5 min of initiation of the reaction in all of the protein fractions (data not shown).

Effect of Temperature on PLD Activity. Temperature is an important variable that affects the quality of various perishable produce items during postharvest storage or during processing. The effect of varying temperature on PLD activity in the microsomal, mitochondrial, and cytosolic fractions was studied to understand the differential effects of temperature on these enzyme preparations. Because the activity of membranous PLD is affected by its membrane environment, changes in the physicochemical properties of the membrane such as phase transitions, changes in gel to liquid crystalline state, and changes in ionic state of the headgroups can affect its activity (Paliyath and Droillard, 1992). By contrast, cytosolic PLD is not normally subjected to such conditions unless it becomes membrane-bound. The temperature was varied from 2.5 to 35 °C, and PLD activity was estimated. The mitochondrial PLD activity did not show any significant change with changing temperature between 15 and 35 °C (Figure 2). However, between 6 and 15 °C, there was a notable increase in activity. Microsomal PLD activity increased with decreasing temperature, especially between 15 and 5 °C. The cytosolic PLD activity followed a similar profile, showing nearly 50% enhancement of its activity at 30 °C, between 15 and 5 °C. Thus, the cytosolic PLD showed the highest degree of promotion in activity between 5 and 15 °C (Figure 2).

Effect of Varying pH on PLD. PLD activity has been reported to be promoted at an acidic pH (Galliard, 1980). Under processing conditions, wounding or blending of the tissue could result in the mixing of intracellular contents that could result in changes in the pH. Again, some of the blending processes could be conducted at an acidic pH. Therefore, the potential effects of changing pH on the activities of microsomal, mitochondrial, and cytosolic PLD were investigated. Citrate buffer in the range of pH 4−7 and Tris-HCl in the pH range of 6−9 were used for these estimations (Figure 3). The mitochondrial PLD retained similar activity throughout the pH ranges tested. Cytosolic and microsomal preparations showed a slight enhancement in activity (10%) in the pH range of 7.5−8.5. Apparently, corn kernel PLD activity does not appear to be affected significantly by changing pH.

Modulation of PLD by Aldehydes and Alcohols. When chilling-sensitive produce items are cooled for storage, especially under low-oxygen atmosphere, the storage conditions induce the anaerobic respiratory pathway leading to the formation of ethanol and acetaldehyde. PLD has a special characteristic termed transphosphatidylation in which the enzyme, in the presence of alcohols, exchanges the phospholipid headgroup for the alcoholic moiety, leading to the production of phosphatidyl alcohol (e.g., phosphatidylethanol, phosphatidylmethanol, etc.). There is a promotion of PLD activity in the presence of alcoholic solvents especially at elevated temperature. It is likely that harvested corn or other chilling-sensitive produce stored under cold, low-oxygen conditions before processing could respire anaerobically, causing the formation of ethanol and acetaldehyde, both of which are off-flavors for processed

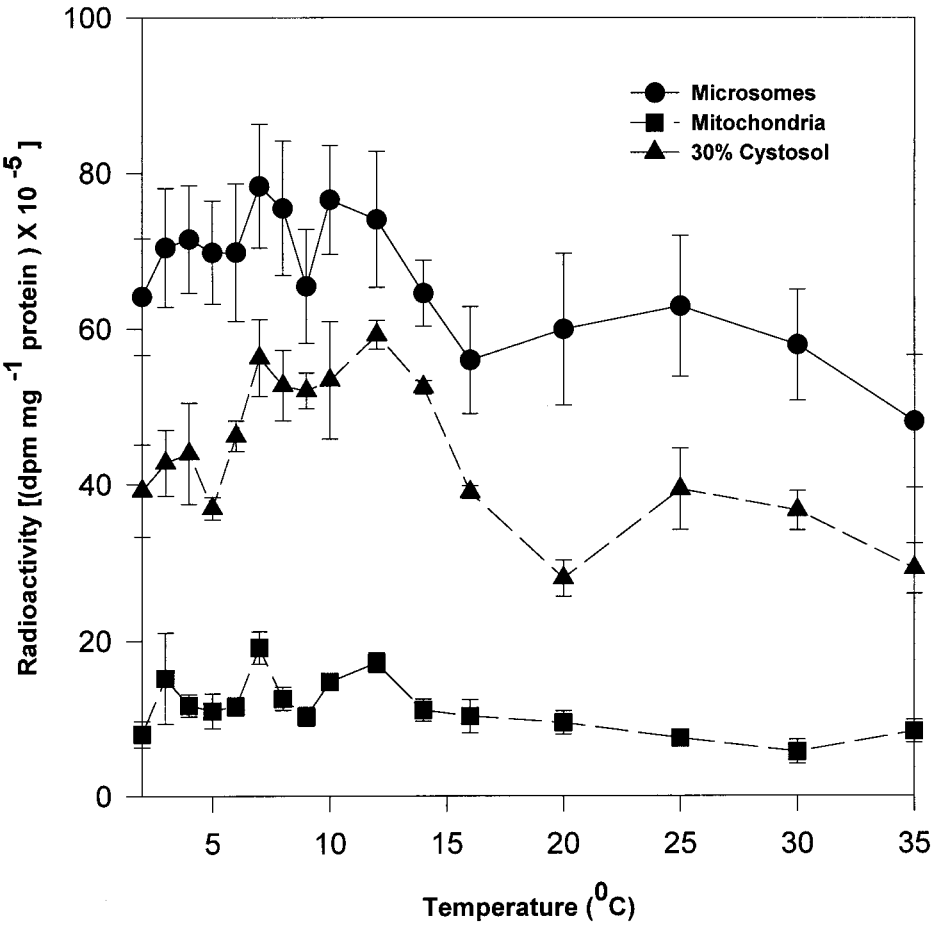

Figure 2. Effect of temperature on PLD activity of the mitochondrial, microsomal, and cytosolic fractions. Membrane and soluble fractions were equilibrated in the assay mixture at appropriate temperatures for 10 min before the reaction was initiated. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min at the indicated temperatures. The values are mean ± SE from three separate estimations.

products. As well, these compounds could also affect PLD activity during processing. Therefore, the effects of ethanol and acetaldehyde on PLD activity were investigated.

Both mitochondrial and microsomal PLD activities were marginally stimulated at 0.25% (v/v) ethanol (Figure 4). Cytosolic PLD also showed a slight stimulation initially followed by a decline in activity (Figure 4). The effects with other longer chain alcohols such as propanol and butanol were similar, although this might not be of any practical relevance under processing or storage conditions (data not shown).

Acetaldehyde is a product of anaerobic metabolism, and off-flavor due to acetaldehyde causes a decline in the quality of the produce. Microsomal, mitochondrial, and cytosolic PLD activities were inhibited by nearly the same level by increasing the content of acetaldehyde in the assay mixture. At 2% v/v, all three forms were inhibited by as much as 80% when compared to the activities in the absence of the aldehyde (Figure 5). Thus, small-chain alcohols and aldehydes that are normal metabolites in perishable produce could affect the membrane deteriorative processes.

Production of long-chain aldehydes such as hexanal and hexenal and alcohols such as hexanol and hexenol is a normal event during senescence or wounding. Catabolism of membrane lipids during senescence or after wounding results in the liberation of linoleic and linolenic acids that serve as substrates for lipoxygenase activity (Paliyath and Droillard, 1992; Gardner, 1995). Subsequent catabolism of fatty acid hydroperoxides leads to the generation of the volatile aldehyde such as hexanal and its reduction product hexanol (Gardner, 1995). Hexanol and hexanoic acid are common components of flavor volatiles in ripening apple fruits (Paliyath et al., 1997). Because of their natural occurrence and abundance in fruits and vegetables, the effect of hexanal and hexanol on PLD activity was investigated. Both hexanal and hexanol inhibited PLD activity by nearly 70% at 0.1% (v/v) in mitochondrial and microsomal preparations (Figures 6 and 7). Cytosolic PLD activity was inhibited to a lesser extent by hexanal and hexanol with inhibitions ranging from 50 to 60% at 0.1% (v/v) (Figures 6 and 7). (These observations on the inhibition of PLD by hexanal and hexanol have been submitted for obtaining a patent in USA/Canada.)

Effect of Metal Ions on PLD Activity. Sodium, potassium, magnesium, and calcium are major ions present in the tissue. Salts of these ions are also added to products during processing. Blending of the produce in the presence of these ions may lead to a stimulation of PLD activity, especially because calcium and magnesium have been reported to promote PLD activity in other systems (Galliard, 1980). To investigate the potential effect of metal ions on PLD activity, enzyme assays were performed in the presence of Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+}. Increasing the concentration of calcium in the assay mixture resulted in different degrees of activation of the microsomal, mitochondrial, and cytosolic PLD activities. Microsomal and cytosolic PLD

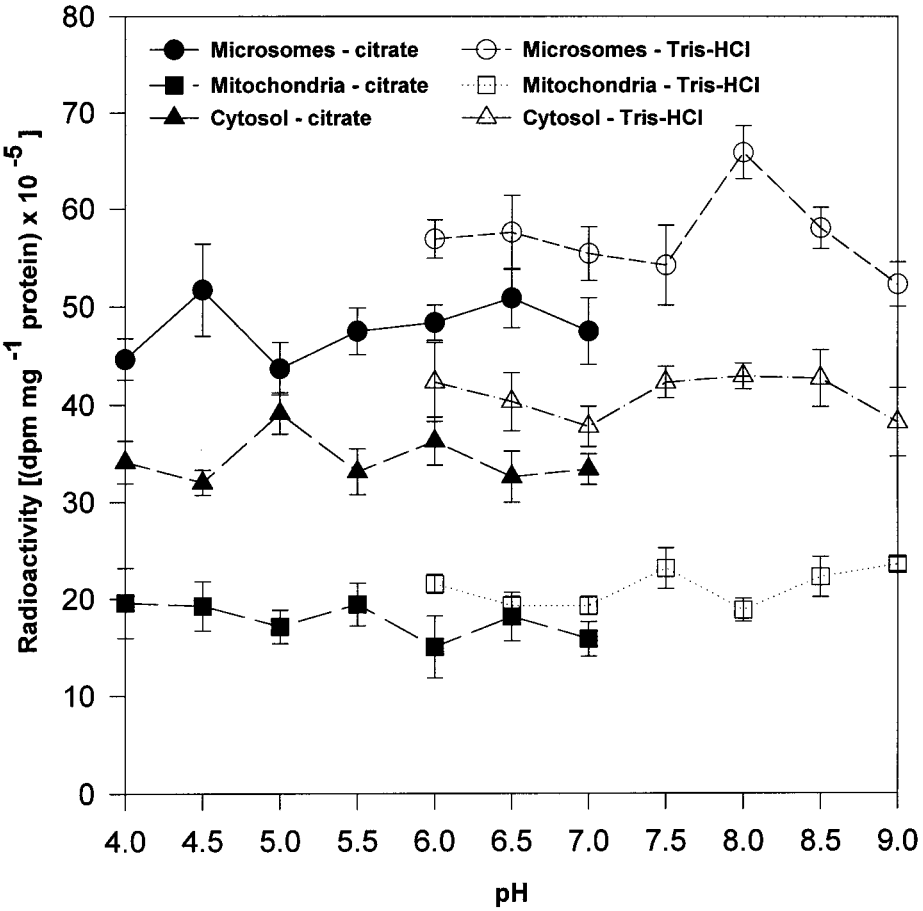

Figure 3. Effect of pH on PLD activity of subcellular fractions of sweet corn kernel. PLD activity in membrane and soluble fractions was assayed at various pH values using Tris-HCl and citrate−phosphate as the buffer systems. Radiolabeled choline released from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min was estimated at various pH values. The values are mean ± SE from three separate experiments.

activities were maximally stimulated by nearly 40−50% at 0.5 mM of added calcium (Figure 8). Mitochondrial PLD showed a marginal stimulation under such conditions. Interestingly, the rate of stimulation was much higher at a low micromolar level of calcium. The assay mixture contained 0.2 mM EGTA, a specific chelator of calcium that lowers the free calcium concentration below micromolar levels (Paliyath and Thompson, 1987). Thus, at 0.2 mM of added calcium (10 μM of free calcium), both microsomal and cytosolic PLD activities were stimulated by nearly 40%. PLD activity was stimulated to a much higher degree in the presence of added magnesium chloride. Maximal stimulation of microsomal and cytosolic PLD was observed at 50 μM magnesium chloride (the level of free magnesium is taken as equal to added magnesium levels, because EGTA is a poor chelator of magnesium at pH 7.0) (Figure 9). Nearly 100% stimulation was obtained for both microsomal and cytosolic PLD at 50 μM magnesium chloride. The activity was reduced slightly with increasing magnesium chloride in the assay mixture (Figure 9).

Monovalent salts such as potassium chloride and sodium chloride did not appear to have any major effects on PLD activity. Microsomal, mitochondrial, and cytosolic PLD activities remained nearly the same in the presence of sodium chloride up to a concentration of 5 mM (Figure 10). Microsomal PLD was inhibited by the addition of KCl up to 5 mM (Figure 11). Both mitochondrial and cytosolic activities were nearly similar with respect to their respective controls (Figure 11).

Partial Purification of PLD. PLD has been reported to exist as several isoforms (Wang et al., 1993; Dyer et al., 1996). This conclusion was reached by the separation of PLD using size exclusion columns or by the expression of PLD genes in experimental cloning systems. All membrane-degrading enzymes exist as soluble and membranous forms (Galliard, 1980), and the rationale for their compartmentation has not been fully established. PLD is reported to be encoded by a single gene (Ueki et al., 1995). Therefore, the existence of cytosolic and membranous PLD defies explanation. It has been reported that the association of PLD with the membrane could occur in a calcium-dependent manner (Ryu and Wang, 1996). Any potential biochemical differences that exist between the microsomal, mitochondrial, and cytosolic forms of PLD need systematic investigation.

To briefly address this question, PLD from mitochondrial and microsomal fractions was isolated by solubilization and subjected to partial purification using DEAE-Sephacel and Sephacryl S-300 gel filtration. Cytosolic PLD was subjected to ammonium sulfate precipitation, and the concentrated fraction was subjected to dialysis and anion exchange chromatography followed by gel filtration. The approximate molecular masses of the major peaks of PLD activity obtained after Sephacryl gel filtration are given in Table 1. Despite their differences in biochemical properties, the different

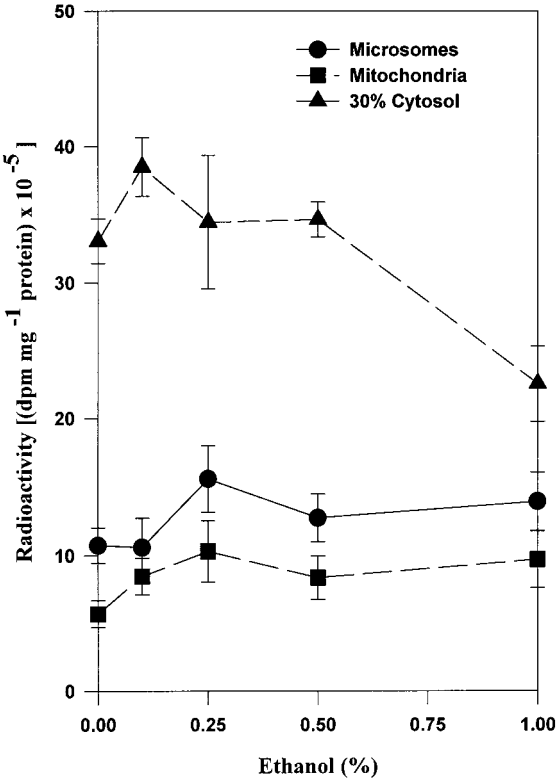

Figure 4. Effect of ethanol on PLD activity. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The values are mean ± SE from three separate experiments.

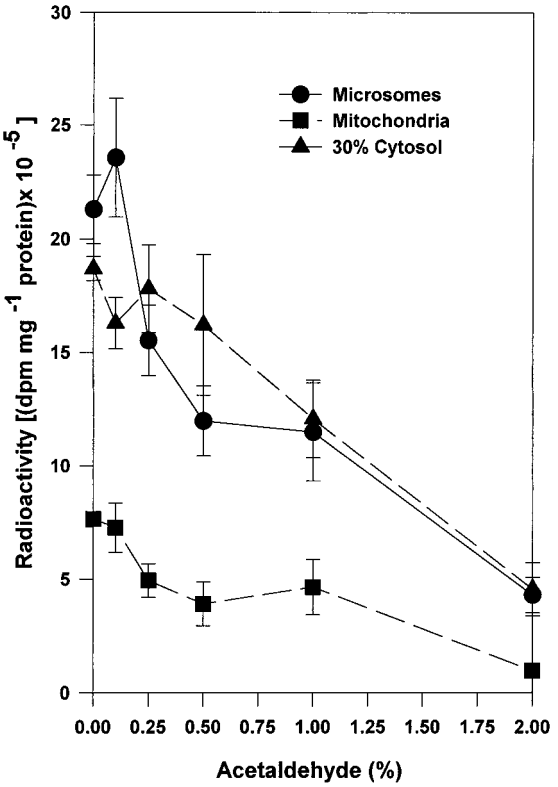

Figure 5. Effect of acetaldehyde on PLD activity of sweet corn kernel membrane and soluble fractions. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The values are mean ± SE from three separate experiments.

isoforms of PLD obtained from the microsomal, mitochondrial, and cytosolic fractions possessed a similar

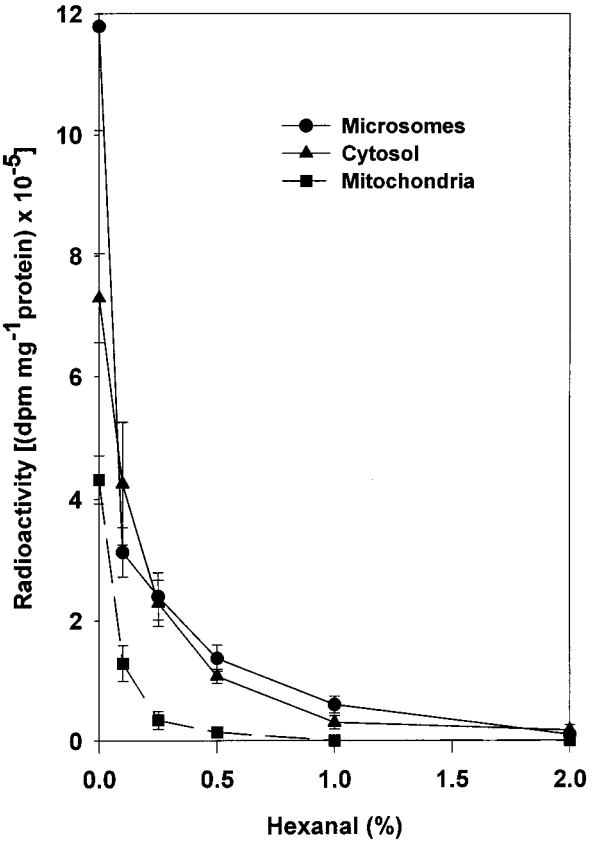

Figure 6. Effect of hexanal on PLD activity of sweet corn kernel membrane and soluble fractions. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The values are mean ± SE from three separate experiments

distribution of molecular masses. Four major isoforms with relative molecular masses of 200, 140−150, 102−116, and 60−66 kDa were observed in all three preparations, the only exception being the mitochondrial fraction in which the 60−66 kDa isoform was undetectable. The smallest isoform in the mitochondrial fraction possessed a relative molecular mass of 108 kDa. During these analyses, we also estimated the relative proportion in the activities of the various isoforms of PLD in the mitochondrial, microsomal, and cytosolic fractions. The distribution of the various isoforms in the mitochondrial fraction show that the 200 kDa isoform is nearly twice that of the other three isoforms on an activity basis (Table 2). In microsomal PLD, the relative distribution among the four isoforms is nearly equal. However, the cytosolic PLD shows the predominant existence of the 200 kDa isoform (3−4 times) as compared to the other low molecular mass isoforms having proportions that are nearly equal (Table 2). If the presence of the various isoforms is a reflection of the in vivo distribution of PLD, then the differences in the relative distribution of the various isoforms may affect the total activity and physicochemical properties of PLD within a given subcellular compartment.

DISCUSSION

PLD activity is central to the maintenance of membrane properties and function because of its involvement in both membrane biogenesis and deterioration. Its activity is regulated by several environmental factors such as heating, chilling, and freezing; hormones such

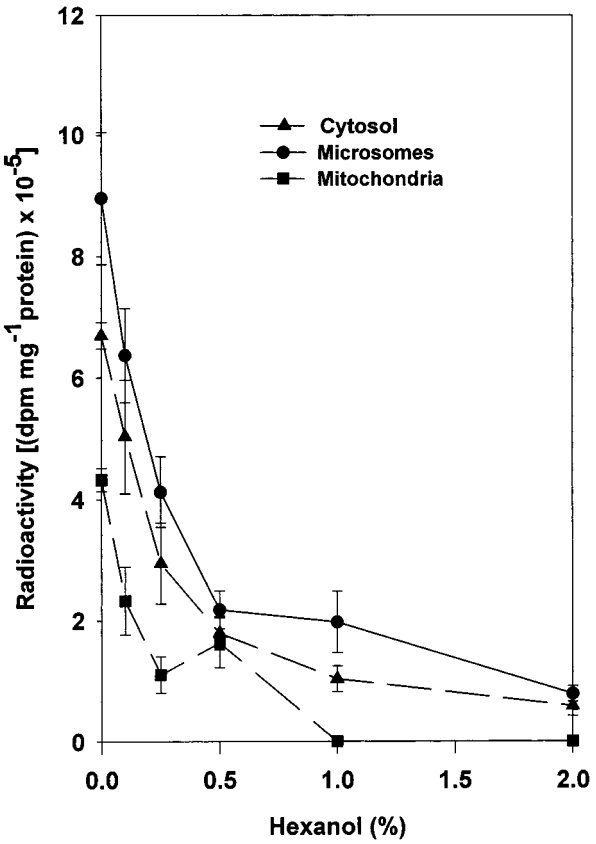

Figure 7. Effect of hexanol on PLD activity of sweet corn kernel membrane and soluble fractions. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The values are mean ± SE from three separate experiments.

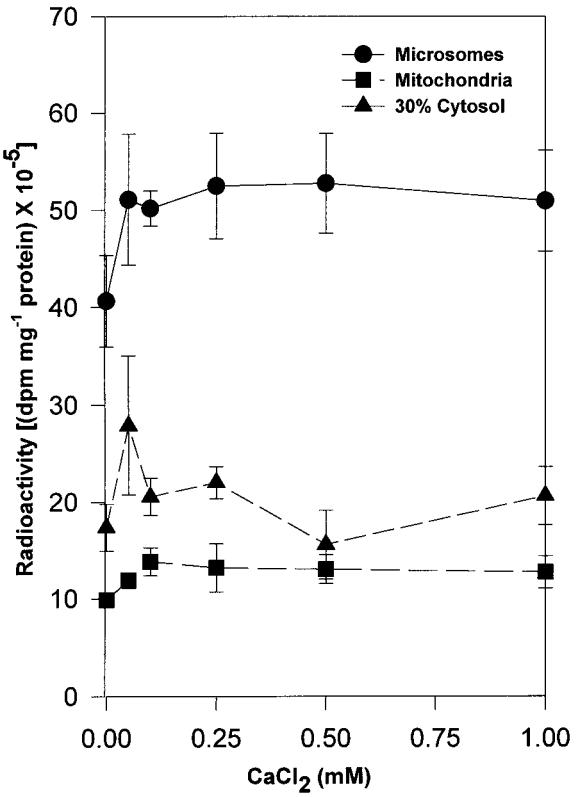

Figure 8. Effect of calcium on PLD activity of sweet corn kernel preparations. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The reaction mixture contained 0.2 mM EGTA, which lowered the free Ca^{2+} levels below micromolar levels in the assay mixture. The free Ca^{2+} concentration levels for added Ca^{2+} concentrations of 100 and 250 μM were 1 and 40 μM, respectively, as determined by a calcium ion electrode (Orion). The values are mean ± SE from four separate experiments.

as auxins, ethylene, cytokinins; ions such as Ca^{2+}, Mg^{2+}, and H^{+}; membrane rigidifying agents such as polyamines; and solvents such as ethanol (Paliyath and Droillard, 1992; Merillon et al., 1995).

We observed PLD activities in both membranous and cytosolic fractions of sweet corn kernel. A similar observation was reported by us earlier in leaf and root tissues of maize seedlings (Pinhero et al., 1998). Even though Brauer et al. (1990) assayed PLD activities in corn root preparation only in 6000*g* and 90000*g* pellets, they did not discount the presence of PLD activity in the 90000*g* supernatant, as evidenced from their results. The existence of cytosolic and membranous forms of PLD has been recognized previously (Yoshida, 1979; Galliard, 1980; Xu et al., 1996). The present study has revealed the differential distribution of various isoforms in the membranous and cytosolic compartments. The cytosol possesses the highest level of the 200 kDa isoform. Because these preparations are only partially purified, we do not know the exact specific activities of these various forms. However, compartmentation of the 200 kDa isoform in cytosol may have an advantage in stabilizing the membrane under stressful conditions. This compartmentation could be advantageous for sweet corn kernel in terms of sustaining low membrane deterioration during long-term cold storage. Processing corn is harvested in bulk and stored at low temperature, if the processing operations cannot handle the volume of produce. Chilling of corn should be rapid to quickly bypass the temperature regime between 15 and 5 °C, at which the activity of PLD is much higher. At lower temperatures, there is also differential regulation of other enzymes involved in membrane lipid degradation. For instance, phosphatidate phosphatase and lipolytic acyl hydrolase activities are considerably lower at 4 °C than PLD activity in tomato microsomal membranes (Todd et al., 1992). If this were to occur in vivo, there would be abundant accumulation of phosphatidic acid in the membrane at low chilling temperatures, which will lead to the breakdown in cellular compartmentation. This will decrease the quality of the produce.

Microsomal and cytosolic fractions of PLD showed optimal activity at pH 8.0, whereas PLD from mitochondria had the optimum activity at a pH around 7.5. It has been reported that PLD exists as different isoforms in castor bean and all these forms showed a pH optima of 6.5 (Dyer et al., 1994). However, Dyer et al. (1996) from their study on PLD from 10 dicots demonstrated that structural heterogeneity of PLD occurs widely in plants. Hence, it could be possible that the PLD isoforms observed in sweet corn could be different from that of castor bean with different catalytic properties. It has been reported that the PLD in mung bean has a pH optimum of around 5.0 (Herman and Chrispeels, 1980), whereas that from citrus callus tissue has a pH optimum of 6.5 (Witt et al., 1987). Similar to the results of microsomal and cytosolic PLD, Imamura and Horiuti (1979) obtained a pH optima of 8.0 for PLD from *Streptomyces chromofuscus*. Apparently, changes in the pH of the processing medium may not significantly affect PLD activity in corn.

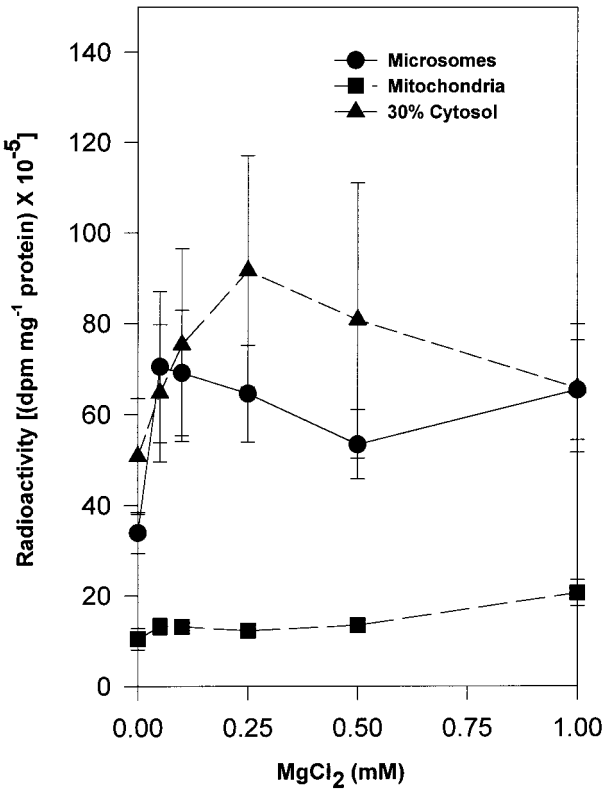

Figure 9. Effect of $MgCl_2$ on PLD activity of sweet corn kernel membrane and soluble fractions. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The data represent mean ± SE from four separate experiments.

Primary alcohols such as ethanol, propanol, butanol, and hexanol and aldehydes such as acetaldehyde and hexanal appear to modulate PLD activity. The transphosphatidylation activity of PLD in the presence of alcohols such as methanol and ethanol is well established (Galliard, 1980). Mitochondrial and microsomal PLD showed a stimulation in their activity in the presence of ethanol. The cytosolic PLD activity appears to be inhibited at ethanol levels >0.5%. Changes in the activity in the presence of propanol or butanol were marginal and may not be significant in vivo. However, PLD activity was inhibited over 75% in all of the preparations by acetaldehyde. A long-chain aldehyde such as hexanal and its corresponding alcohol, hexanol, also showed potent inhibition of PLD activity. These effects could implicate other means of natural regulation of PLD activity. Acetaldehyde, which is inhibitory to PLD, is an undesirable byproduct of anaerobic breakdown of sugars. Inhibition of PLD by acetaldehyde could serve as a check for inhibiting membrane lipid degradation under stressful conditions, under which anaerobic pathways could be activated (e.g., chilling). Similarly, hexanal and hexanol are byproducts of lipoxygenase and associated enzyme activities in plant systems, which are part of the autocatalytic membrane deteriorative pathways (Paliyath and Droillard, 1992; Gardner, 1995). Thus, inhibition of PLD by hexanal and hexanol could serve as a check point for arresting membrane lipid degradation. These components are also part of the natural flavor ingredients and could be of potential use in preventing excessive membrane lipid degradation during storage or under processing conditions. In animal systems, hydroxyhexenal and other long-chain aldehydes are promoters of PLD (Natarajan et al., 1993).

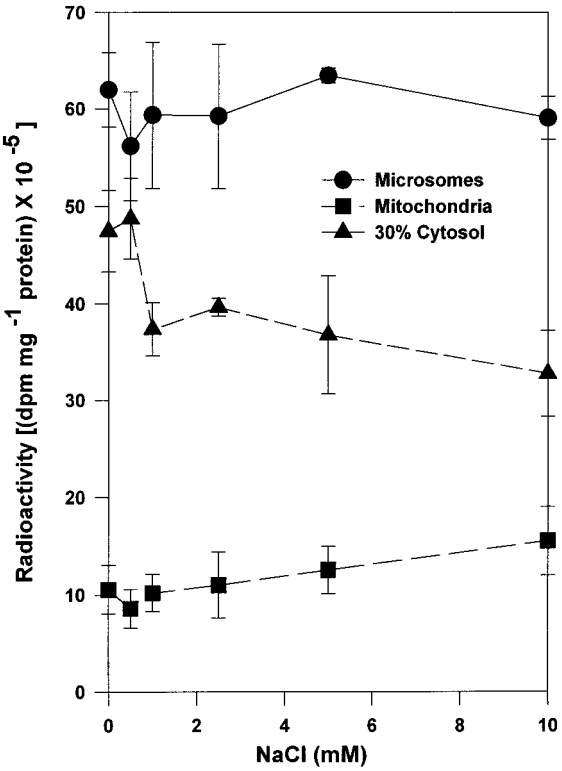

Figure 10. Effect of NaCl on PLD activity of sweet corn kernel preparations. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The data represent mean ± SE from three separate experiments.

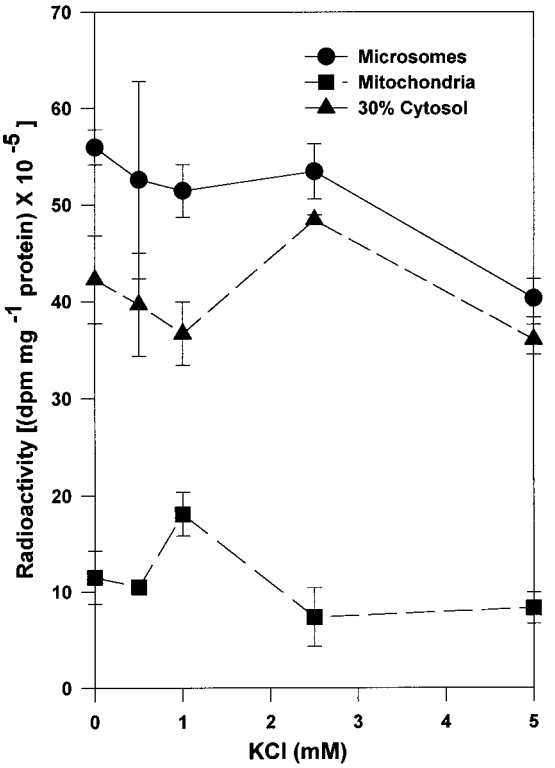

Figure 11. Effect of KCl on PLD activity of sweet corn kernel preparations. PLD activity was measured by the release of radiolabeled choline from 16:0/16:0 phosphatidylcholine during an incubation period of 10 min. The results represent mean ± SE from three independent experiments.

The differences in their mode of action may stem from the structural differences between plant and animal

Table 1. Distribution of Molecular Forms of PLD in Sweet Corn Kernel[a]

major peak	molecular mass (kDa)		
	cytosol	mitochondria	microsomes
1	200	200	200
2	150	140	140
3	116	116	102
4	66	108	60

[a] PLD from the mitochondrial, microsomal, and cytosolic fractions was independently isolated and subjected to anion exchange on DEAE-Sephacel and size exclusion chromatography on Sephacryl S-300 HR. Peaks of PLD activity eluting from the Sephacryl column were determined. Relative molecular mass of protein eluted at the peak was determined using a Sigma molecular mass standard mixture.

Table 2. Relative Proportion of PLD Activity in the Major Peaks Eluted during Purification Using Sephacryl Gel Filtration[a]

protein fraction	relative proportion of PLD activity
cytosol	4.6:1.2:1:1.3 (200:150:116:66)
microsomes	1.3:1.4:1.8:1 (200:140:102:60)
mitochondria	2.3:1:1.02:1 (200:140:116:108)

[a] PLD activity was measured from the fractions collected during gel filtration through a Sephacryl S-300 HR column, and the activity under the peak was determined. The peak with the smallest area is considered as 1 and others are expressed as a multiple of this value.

PLDs and their physiological function. Other compounds with PLD-inhibitory activity have been reported in plants. Lysophosphatidylethanolamine, which is a natural constituent of plant membranes, has been identified as a potent inhibitor of PLD (Ryu et al., 1997) with application in horticulture industries.

Ca^{2+} ions are known to activate PLD at low micromolar and millimolar levels (Paliyath and Thompson, 1987; Brauer et al., 1990). In leaf and root tissues of maize seedlings, PLD activity was not stimulated by either calcium or magnesium (Pinhero et al., 1998). In corn kernels, PLD activity was marginally stimulated by calcium ions. The stimulation by magnesium ions appeared to be slightly higher than that of calcium ions. As well, the microsomal and cytosolic PLD was activated to a higher extent than mitochondrial PLD. Thus, processing conditions having high levels of magnesium and calcium can lead to increased PLD. By contrast, monovalent salts such as sodium chloride or potassium chloride did not affect PLD activity substantially.

The existence of PLD isoforms with relative molecular masses of 200, 140−150, 102−116, and 60−66 kDa is an interesting feature that may have regulatory effects on phospholipid catabolism. Purification of PLD from different sources showed high variability in its molecular weights. Molecular weights of 200, 112.5, and 90.5 kDa have been reported in different tissues such as peanut seed (Heller, 1978), Savoy cabbage leaves (Allgyer and Wells, 1979), and citrus callus tissues (Witt et al., 1987). Wang et al. (1993) identified a 92-kDa protein as PLD. Dyer et al. (1994) reported three PLD isoforms from castor bean, and the molecular masses of these variants were estimated by size exclusion chromatography to be 330, 270, and 230 kDa. It is likely that in corn kernels PLD could exist in an oligomeric form, with the molecular mass of the monomer being in the range of 60−66 kDa. Further studies are required to delineate the molecular nature of PLD in corn kernels. As well, our studies also show that the relative distribution of various isoforms could vary between the mitochondrial, microsomal, and cytosolic preparations. The cytosolic preparation had a 3−4-fold higher abundance of the 200 kDa isoform as compared to the microsomal membranes. As well, on a total activity basis, cytosol possessed the highest level of PLD activity. Whether this reflects the true in situ distribution or is a result of an extraction anomaly is not clear at present. However, the differences in the distribution of various isoforms and the potential differences in their biochemical and physical properties may determine the differential susceptibility of the subcellular structures to degradation under various physiological and processing conditions.

ABBREVIATIONS USED

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium; EGTA, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; PLD, phospholipase D.

LITERATURE CITED

Allgyer, T. T.; Wells, M. A. Phospholipase D from savoy cabbage: Purification and preliminary kinetic characterization. *Biochemistry* **1979**, *18*, 5348−5353.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

Brauer, D.; Nungesser, E.; Maxwell, R. J.; Schubert, C.; Tu, S. I. Evidence for and subcellular localization of a Ca-stimulated phospholipase D from maize roots. *Plant Physiol.* **1990**, *92*, 672−678.

Cockfort, S. Phospholipase D: Regulation by GTPases and protein kinase C and physiological relevance. *Prog. Lipid Res.* **1997**, *35*, 345−370.

Dyer, J. H.; Ryu, S. B.; Wang, X: Multiple forms of phospholipase D following germination and during leaf development of castor bean. *Plant Physiol.* **1994**, *105*, 715−724.

Dyer, J. H.; Zheng, S.; Wang, X. Structural heterogeneity of phospholipase D in 10 dicots. *Biochim. Biophys Res. Commun.* **1996**, *221*, 31−36.

Edwards, G. E.; Gardestrom, P. Isolation of mitochondria from leaves of C_3, C_4 and crassulacean acid metabolism plants. *Methods Enzymol.* **1987**, *148*, 421−433.

Exton, J. H. Phospholipase D: Enzymology, mechanisms of regulation, and function. *Physiol Rev.* **1997**, *77*, 303−320.

Galliard, T. Degradation of acyl lipids: Hydrolytic and oxidative enzymes. In *The Biochemistry of Plants, A Comprehensive Treatise*; Stumpf, P. K., Conn, E. E., Eds.; Academic Press: New York, 1980; Vol. 4, Chapter 3.

Gardner, H. W. Biological roles and biochemistry of the lipoxygenase pathway. *HortScience* **1995**, *30*, 197−205.

Harris, W. E.; Knutson, C. M.; Stahl, W. L. A fluorescent method for study of cabbage phospholipase D activity. *Plant Physiol. Biochem.* **1995**, *33*, 389−398.

Heller, M. Phospholipase D. *Adv. Lipid Res.* **1978**, *16*, 267−326.

Herman, E. M.; Chrispeels, M. J. Characteristics and subcellular distribution of phospholipase D and phosphatidic acid phosphatase in mung bean cotyledons. *Plant Physiol.* **1980**, *66*, 1001−1007.

Imamura, S.; Horiuti, Y. Purification of *Streptomyces chromofuscus* phospholipase D by hydrophobic affinity chromatography on palmitoyl cellulose. *J. Biochem.* **1979**, *85*, 79−95.

Kates, M. Hydrolysis of lecithin by plant plastid enzymes. *Can. J. Biochem. Physiol.* **1955**, *33*, 575−589.

McCormac, D. J.; Todd, J. F.; Paliyath, G.; Thompson, J. E. Modulation of bilayer fluidity affects lipid catabolism in microsomal membranes of tomato fruits. *Plant Physiol. Biochem.* **1993**, *31*, 1−8.

Merillon, J. M.; Filali, M.; Duperon, P.; Montagu, M.; Chenieux, J. C.; Rideau, M. Effect of 2,4-dichlorophenoxyacetic acid and habituation on lipid and protein composition of microsomal membranes from periwinkle cell suspensions. *Plant Physiol. Biochem*. **1995**, *30*, 443−451.

Natarajan, V.; Scriber, W. M.; Taher, M. M. 4-Hydroxy nonenal a metabolite of lipid peroxidation activates phospholipase D in vascular endothelial cells. *Free Radicals Biol. Med*. **1993**, *15*, 365−375.

Paliyath, G.; Thompson, J. E. Calcium and calmodulin-regulated breakdown of phospholipid by microsomal membranes from bean cotyledons. *Plant Physiol.* **1987**, *83*, 63−68.

Paliyath, G.; Droillard, M. J. The mechanisms of membrane deterioration and disassembly during senescence. *Plant Physiol. Biochem*. **1992**, *30*, 789−812.

Paliyath, G.; Whiting, M. D.; Stasiak, M. A.; Murr, D. P.; Clegg, B. S. Volatile Production and fruit quality during development of superficial scald in Red Delicious apples. *Food Res. Int*. **1997**, *30*, 95−103.

Pinhero, R. G.; Paliyath, G.; Yada, R. Y.; Murr, D. P. Modulation of phospholipase D and lipoxygenase activities during chilling. Relation to chilling tolerance of maize seedlings. *Plant Physiol. Biochem*. **1998**, *36*, 213−224.

Quarles, R. H.; Dawson, R. M. C. Distribution of phospholipase D in developing and mature plants. *Biochem. J*. **1969**, *112*, 787−794.

Ryu, S. B.; Wang, X. Expression of phospholipase D during castor bean leaf senescence. *Plant Physiol*. **1995**, *108*, 713−719.

Ryu, S. B.; Wang, X. Activation of phospholipase D and the possible mechanism of activation in wound-induced lipid hydrolysis in castor bean leaves. *Biochim. Biophys. Acta* **1996**, *1303*, 243−250.

Ryu, S. B.; Zheng, L.; Wang, X. Change in phospholipase D expression in soybeans during seed development and germination. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1171−1176.

Ryu, S. B.; Karlson, B. H.; Ozgen, M.; Palta, J. P. Inhibition of phospholipase D by lysophosphatidylethanolamine, a lipid derived senescence-retardant. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 12717−12721.

Takano, K.; Kamoi, I.; Obara, T. Properties and degradation of rice bran spherosome. *J. Jpn. Soc. Food Sci.* **1989**, *36*, 468−474.

Todd, J. F.; Paliyath, G.; Thompson, J. E. Effect of chilling on the activities of lipid-degrading enzymes in tomato fruit microsomal membranes. *Plant Physiol. Biochem*. **1992**, *30*, 517−522.

Ueki, J.; Morioka, S.; Komari, T.; Kumashiro, T. Purification and characterization of phjsopholipase D (PLD) from rice (*Oryza sativa* L.) and cloning of cDNA for PLD from rice and maize (*Zea mays* L.). *Plant Cell Physiol*. **1995**, *36*, 903−914.

Voisine, R.; Vezina, L.-P.; Willemot, C. Modification of phospholipid catabolism in microsomal membranes of γ-irradiated cauliflower (*Brassica oleracea* L.). *Plant Physiol.* **1993**, *102*, 213−218.

Wang, X.; Dyer, J. H.; Zheng, L. Purification and immunological analysis of phospholipase D from germinating castor bean endosperm. *Arch. Biochem. Biophys.* **1993**, *306*, 486−494.

Witt, W.; Yelenosky, G.; Mayer, R. T. Purification of phospolipase D from citrus callus tissue. *Arch. Biochem. Biophys.* **1987**, *259*, 164−170.

Xu, L.; Paulsen, A. Q.; Ryu, S. B.; Wang, X. Intracellular localization of phospholipase D in leaves and seedling tissues of castor bean. *Plant Physiol.* **1996**, *111*, 101−107.

Yoshida, S. Freezing injury and phospholipid degradation in vivo in woody plant cells. Subcellular localization of phospholipase D in living bark tissues of the black locust tree (*Robinia pseudoacacia L.*). *Plant Physiol.* **1979**, *64*, 241−246.

Received for review September 24, 1998. Revised manuscript received April 15, 1999. Accepted April 21, 1999. This research was conducted with financial support from the Ontario Ministry of Agriculture, Food and Rural Affairs and the Natural Sciences and Engineering Research Council of Canada.

JF981048N