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# Decreased Membrane Integrity in Castor Bean Mitochondria by Hydrogen Peroxide. Evidence for the Involvement of Phospholipase D.

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**Purified mitochondria from germinating castor bean (*Ricinus communis* L.) endosperm was treated with hydrogen peroxide ($H_2O_2$), active oxygen form, in order to investigate the extent of membrane degradation. Incubation of mitochondria with micromolar concentrations (50-200 μM) of $H_2O_2$ resulted in a concentration-dependent loss of membrane proteins. During this process extensive loss of lipid-phosphate content was also observed in mitochondrial membranes. When L-3-phosphatidyl[2-$^{14}$C]ethanolamine was added to the mitochondrial membranes as an exogenous substrate, the level of radioactivity in the water-soluble fraction was markedly enhanced with increasing concentration of $H_2O_2$. Analysis of the water-soluble products formed during the metabolism of ethanolamine-labelled phosphatidylethanolamine by mitochondrial membranes from castor bean indicates that this loss of lipid-phosphate is attributable to action of phospholipase D. Direct measurement of mitochondrial phospholipase D indicated that the activity of enzyme was remarkably stimulated by calcium ion or sodium dodecylsulfate (SDS). The optimum concentrations for enzyme stimulation were 25 and 0.5 mM for calcium ion and SDS in the reaction mixture, respectively. The substrate specificity of phospholipase D was determined by comparing various classes of exogenous phospholipids, added in the form of sonicated vesicles, as substrates. The phospholipase D exhibited preference for phosphatidylethanolamine. Taken together, our results suggest that increase of mitochondrial phospholipase D activity may be a key event leading to accelerated membrane deterioration following active oxygen attack.**

*Keywords*: active oxygen species, hydrogen peroxide, mitochondrial membranes, phospholipids, phospholipase D

The formation of hydrogen peroxide ($H_2O_2$), active oxygen species such as single oxygen, superoxide anion, and hydroxyl radical, is a fundamental consequence of aerobic metabolism. In addition, $H_2O_2$ is commonly synthesized in response to various environmental stimuli (Sutherland, 1991). Such factors as UV light, herbicides, drought, temperature stress, and intensive light are known to induce $H_2O_2$ formation in plants (Scandalios, 1993). Recently, particular interest has been the involvement in plant defense responses (Mehdy, 1994). For example, the release of active oxygen species from cell suspensions of cotton, tobacco, and soybean only minutes after the addition of crude extract from the pathogenic fungus *Verticillium dahliae* was reported (Apostol *et al.*, 1989). However, the exact roles played by $H_2O_2$ in these various physiological processes are still unclear.

Reactive oxygen species are produced in various subcellular compartments or organelles. $H_2O_2$ is produced through the primary production of superoxide anion ($O_2^-$) and its subsequent dismutation. This reaction is catalyzed by superoxide dismutase (SOD) located in the cytosol, chloroplast, or mitochondria. The production of superoxide anion and $H_2O_2$ in the mitochondria of germinating seeds has been reported by Puntarulo *et al.* (1988) using soybean embryonic axes. It was suggested that up to 2% of mitochondrial oxygen consumption is involved in $H_2O_2$ generation and mitochondria are the most important

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cytosolic source of $H_2O_2$ in germinating seeds (Puntarulo *et al.*, 1991).

Progressive decline in membrane phospholipid content is a general feature of membrane deterioration in free radical treated plant tissue (Voisine *et al.*, 1991). It has been reported that free radical attack on membrane lipid is the main cause of membrane damage by the active oxygen. The reaction of free radical with polyunsaturated fatty acids in acyl chains leads to formation of lipid peroxidation in membranes (Mead, 1976). Hydrogen peroxide can generate hydroxyl radical (OH) through the iron catalyzed Haber-Weiss reaction (Bowler *et al.*, 1992). It is also possible that decreased synthesis of phospholipids, increased phospholipids catabolism or both may be involved in the membrane degradation process by active oxygen species. Recently, it has been reported in membrane degradation after gamma irradiation that lipid catabolism is mediated, at least part, by enzymes including phospholipase, phosphatidic acid phosphatase, or lipolytic acyl hydrolase (Voisine *et al.*, 1991). The manifestation of this catabolism includes a selective depletion of fatty acid and loss of head group from membrane phospholipids.

Phospholipase D (PLD; EC 3.1.1.4), an enzyme that catalyzes hydrolysis of glycerophospholipids at the terminal phosphodiester bond, generates phosphatidic acid and a polar head group. In animal system, activation of PLD occurs in response to many stimuli such as growth factors, hormones, and platelet activating factors (Billah, 1993). Recent studies suggest that hydrolysis products of PLD act as an intracellular second messenger in many different cell types (Fukami and Takenawa, 1992; Perky *et al.*, 1992). PLD occurs widely in the plant kingdom as many different forms (Heller, 1978). Although PLD has recently been cloned from castor bean (Wang *et al.*, 1994) and arabidopsis (Pappan *et al.*, 1997), its physiological role in the plant kingdom is still not much known. The various PLD forms from plant have been reported from gamma-irrated cauliflower (Voisine *et al.*, 1993), senescence of flower petals (Paliyath *et al.*, 1987), and germinating pea embryos (Nola and Mayer, 1986). PLD-mediated hydrolysis is a first step in membrane deterioration in these physiological processes. However, whether change of these PLD activities predominantly serves a metabolic or signal transduction role in the plant remains unresolved.

The mechanism of underlying physiological effects of active oxygen in mitochondrial membrane is not well understood. In present study, the mechanism of membrane degradation by active oxygen attack has been examined. We report evidence indicating that PLD activity mediates the loss of membrane phospholipids during mitochondrial membrane degradation by $H_2O_2$.

## MATERIALS AND METHODS

### Chemicals

L-3-phosphatidyl[2-$^{14}$C]ethanolamine, 1,2-dioleoyl (54 mCi/mmol) was purchased from Amersham. Nonradioactive phospholipids were obtained from Sigma Chemical Co. The sucrose was from Fisher Co. All other organic reagents were obtained from Sigma Chemical Co. The TLC plates were obtained from Merck Co.

### Plant Material

Seeds of castor bean (*Ricinus communis* L. var. Hale) endosperm were removed from their seed coats, surface sterilized in 10% bleach (5.25% hypochlorite) for 90 sec, and planted in moist vermiculite. They were germinated and grown at 30°C for 3 days in the dark.

### Tissue Homogenization

This procedure was similar to the methods described previously (Moore *et al.*, 1973). Thirty endosperm halves were removed from 3-day-old seedlings, rinsed in distilled water, and placed in a petri dish on ice. The endosperm halves were chopped for 15 min with a razor blade in 10 mL of homogenization medium containing 150 mM Tricine buffer (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.15 M sucrose. The crude homogenate was filtered through four layers of cheesecloth.

### Mitochondria Isolation

Five mL of filtered homogenate were layered onto the top of a gradient contained in a 37.5 mL centrifuge tube. The gradient was composed of: (a) a 2.0 mL cushion of 2.25 M sucrose, (b) 20 mL of sucrose solution decreasing linearly in concentration from 2.25 M to 1.0 M, and (c) a 10 mL layer of 0.6 M sucrose. The gradients were centrifuged at 53,000 g in a Kontron ultracentrifuge for 3 hours and the mitochondrial fraction was collected. Mitochondrial purity was monitored by using criteria as described

previously (Marcus *et al.*, 1991). Lack of significant contamination of the cytosol, mitochondria, glyoxysome, and endoplasmic reticulum fractions both by one another and by proteins of other cell compartments was confirmed by assaying enzyme marker. Thus, the specific activity of fumarase (Racker, 1950), a marker of mitochondria was 13.5± 1.5 μmol/min per mg protein in the mitochondria compared with 0.01±0.005 μmol/min per mg protein in cytosol (0.1%). The specific activity of pyrophosphate fructose-6-phosphate 1-phosphotransferase (Morigasaki *et al.*, 1990), a marker of cytosol was 0.011±0.009 μmol/min per mg protein in mitochondria or less than 0.1% of that of cytosol (10.9±0.8 μmol/min per mg protein). The antimycin A-resistant cytochrome c reductase (Lord *et al.*, 1973), an endoplasmic reticulum marker and catalase (Luck, 1965), a glyoxysomal marker, were not detected in mitochondrial fraction.

### $H_2O_2$ Treatment

Aliquots (300-400 μg of protein) of mitochondria were incubated with 2 mL of 150 mM Tris buffer (pH 7.5) containing 0.25 M sucrose and various concentrations of $H_2O_2$ at room temperature. After 20 min incubation, the mixtures were chilled and centrifuged at 100,000 g at 4°C for 60 min. The mitochondrial pellet was resuspednded in 150 mM Tris buffer (pH 7.5) and the susupension was immediately used for lipid extraction. Membrane proteins were determined by Bradford procedure with BSA as standard (Bradford, 1976).

### Phospholipid Analysis

After incubation mitochondria with $H_2O_2$, total lipids were extracted by a method based on the one described in Bligh and Dyer (1959). The final chloroform extract was dried under nitrogen and the lipid residues were redissolved in chloroform: methanol (2:1, v/v) for application to 250 μm silica gel G plates. Phospholipids were separated by two-dimensional chromatography (Hoevet *et al.*, 1968) and identified by using authentic standards. Spots were visualized with iodine vapors and scraped from the plate to measure the amount of phosphorus or radioactivity. Phosphorus was estimated by the method of Rouser *et al.* (1970).

### Analysis of Phospholipid Degradation

The capability of mitochondria fraction to breakdown of phospholipids was determined as described previously (Paliyath *et al.*, 1987). The basic reaction mixture contained 50 mM Hepes, pH 7.0, 4 KBq of L-3-phosphatidyl[2-$^{14}$C]ethanolamine, 1,2-dioleoyl, and 600 μg of $H_2O_2$-treated mitochondrial proteins. The reaction mixture was incubated 30°C for 30 min and then stopped by addition of chloroform: methanol (2:1, v/v) solution. After extraction, an aqueous fraction was used for anion exchange analysis or for determining radioactivity by liquid scintillation counting and a lipid fraction was used for TLC. The separation of phosphatidylethanolamine (PtdEtn) metabolites was performed by modified method of Dowex-50 $WH^+$ column chromatography (Cook and Wakelam, 1989). The column was initially washed with 5 mL water and then run through material contained glycerophosphorylethanolamine. Another washing with 20 mL of water eluted ethanolamine phosphate. Finally ethanolamine was eluted by 20 mL of 1 M HCl.

### PLD Activity Assay

PLD activity was assayed as described earlier (Dyer *et al.*, 1994). The assay mixture (0.5 mL) contained 100 mM Mes-NaOH (pH 6.5), 0.4 μM of L-3-phosphatidyl[2-$^{14}$C]ethanolamine, 1,2-dioleoyl, and 50 μg of 100 μM $H_2O_2$-treated mitochondrial proteins. Calcium and SDS were added to assay mixture as specified. The reaction mixture was incubated 30°C for 30 min. In substrate specificity experiments, nonradioactive phospholipids, phosphatidylcholine (PtdCho), PtdEtn and phosphatidylglycerol were derived from egg yolk, phosphatidylinositol was from soybean and phosphatidylserine was from bovine brain. Each phospholipid was emulsified in 1 mL of water by sonication at room temperature and added in reaction mixture.

## RESULTS

### Degradation of Mitochondrial Membranes by $H_2O_2$

To investigate the effect of $H_2O_2$ on the mitochondrial membrane integrity, we isolated mitochondria from the endosperm of germinating castor beans - a tissue long used for cell fraction studies (Donaldson and Beevers, 1977) and checked the contamination using marker enzymes (details in Materials and Methods). First the change of a mitochondrial membrane protein content upon $H_2O_2$ treatment was examined. As shown in Fig. 1, the

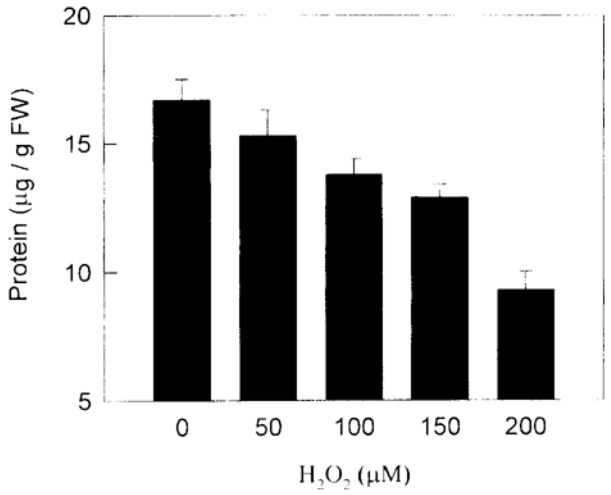


**Fig. 1.** Change in protein content of mitochondrial membranes by treatment of various concentrations of $H_2O_2$. Mitochondria were incubated with $H_2O_2$ at various concentrations in 150 mM Tris buffer, pH 7.5, containing 0.25 M sucrose at room temperature. After 20 min incubation, amounts of mitochondrial membranes protein were measured as described in Materials and Methods. Each point is the mean ± SE ($n$=4 to 6).

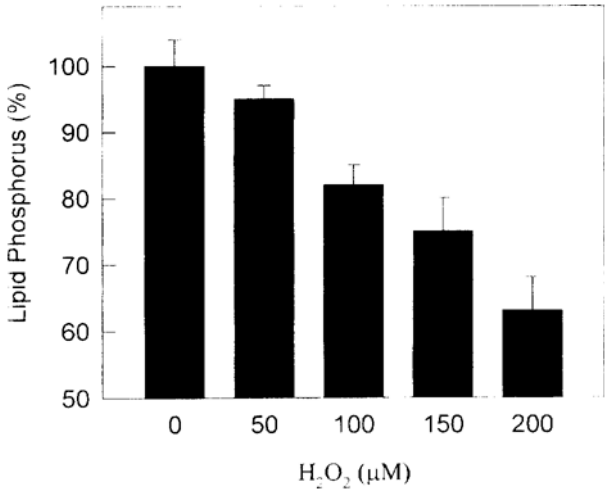


**Fig. 2.** Loss of mitochondrial membrane lipid phosphorus by treatment of various concentrations of $H_2O_2$. The reaction mixture consisted of mitochondria with $H_2O_2$ at various concentrations in 150 mM Tris buffer, pH 7.5, containing 0.25 M sucrose. Incubation was carried out at room temperature. After 20 min incubation, amounts of mitochondrial membranes phosphorus were measured as described in Materials and Methods. Values are expressed as percentage of the control (untreated mitochondria). Value (nmol/mg protein) at control was 550±9 nmol. Data represent the mean ± SE ($n$=4 to 6).

treatment of mitochondria with $H_2O_2$ resulted in a concentration-dependent loss of membrane protein. Approximately 45% of the membrane protein were lost at 200 μM of $H_2O_2$ treatment. Loss of membrane protein indicates a significant alteration of composition of the membranes. Therefore, extensive depletion of membrane protein could contribute to membrane destabilization and degradation.

Loss of lipid phosphate reflecting phospholipid breakdown is perhaps the most characteristic feature of membrane deterioration and has been demonstrated for variety of plant tissues (McKersie *et al.*, 1988). To determine whether phospholipid breakdown occurred by $H_2O_2$, the content of membrane phospholipid was monitored. Fig. 2 shows that the level of phospholipid degradation in mitochndrial membrane was accelerated with increasing concentration of $H_2O_2$. Phospholipids decreased by 35% in the 200 μM $H_2O_2$-treated mitochondria compared to control (without $H_2O_2$ treatment). Our results indicated that change in protein content was associated with an alteration in membrane phospholipid content. The depletion of protein and phospholid led thus to a major compositional alteration in the mitochondrial membranes. It is noteworthy that there was greater loss of mitochondrial membrane protein (45% of control) than the loss of phospholipid content (35% of control) in the 200 μM $H_2O_2$-treated mitochondria.

## Identification of PLD Activity

Because treatment of $H_2O_2$ in mitochondrial membranes induced the breakdown of membrane phospholipids, we considered the possibility that lipid-degradation enzyme activity might be responsible for change of phospholipid content in $H_2O_2$-treated mitochondrial membranes. Mitochondrial membranes proved capable of catalyzing the release of radiolabelled ethanolamine from the lipid phase when phosphatidyl[$^{14}$C]ethanolamine was added to the membranes as an exogenous substrate. As shown in Fig. 3, the treatment of $H_2O_2$ enhanced the rate of degradation of radiolabelled PtdEtn. This could be achieved by the action of phodpholipase C (PLC), PLD or lipolytic acyl hydrolase, which form water-soluble, free phosphorylethanolamine, ethanolamine or glycerophosphorylethanolamine, respectively. In order to distinguish among these possibilities, the metabolic products of ethanolamine-labelled PtdEtn were analyzed by a Dowex-50 $WH^+$ anion exchange chromatography; all of the water-soluble radioactivity eluted with ethanolamine, the expected product of PLD (Fig. 4). No activity was found in the other possible degradation products such as the PLC product

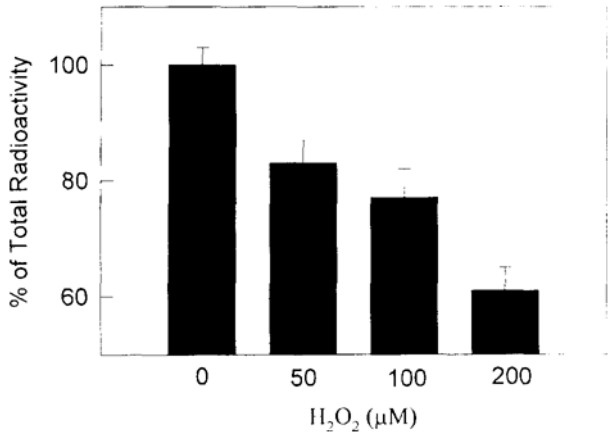


**Fig. 3.** Breakdown of radiolabelled phosphatidyl[2-$^{14}$C] ethanolamine by various concentrations of $H_2O_2$-treated mitochondrial membranes. The reaction mixture consisted of 100 mM Mes/NaOH (pH 6.5), 25 mM $Ca^{2+}$, 50 μg of various concentrations $H_2O_2$-treated mitochondrial proteins, and radiolabelled PtdEtn. The reaction mixture was incubated for 30 min at 30°C. Data represent the mean ± SE ($n$=3).

phosphorylethanolamine, or the acyl hydrolase product glycerophosphorylethanolamine. In order to determine whether phospholipase A is involved in the phospholipid breakdown, lipid fraction from the same sample was separated by TLC. Lyso-PtdEtn, an expected phospholipase A product, was not detected in this experiment (data not shown). Combining all these data indicates that the degradation of phospholipid by $H_2O_2$ in mitochondrial membranes is caused by PLD.

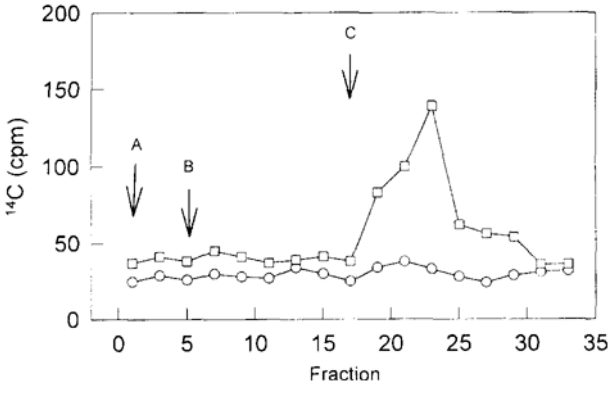


**Fig. 4.** Analysis of water-soluble metabolite products of phosphatidyl[2-$^{14}$C]ethanolamine on mitochondrial membranes with (square) or without (circle) treatment by 100 μM $H_2O_2$ followed by Dowex-50 $WH^+$ anion exchange chromatography. Elution position, glycerophosphorylethanolamine (A); ethanolamine phosphate (B); ethanolamine (C).

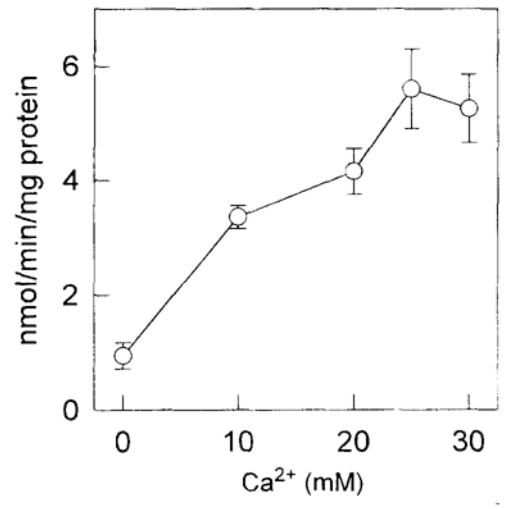


**Fig. 5.** Effects of various concentrations of $Ca^{2+}$ on mitochondrial PLD activities. Enzyme activities were assayed in 100 mM Mes/NaOH (pH 6.5) buffer for 30 min as described in Materials and Methods. The activity of PLD was measured by release of ethanolamine from phosphatidyl[2-$^{14}$C]ethanolamine. Data represent the mean ± SE ($n$=3).

## Properties of PLD

The factors affecting activity of PLD in mitochondria were examined. Boiled mitochondria showed no enzyme activity (data not shown). Several previous studies (McCormac *et al.*, 1993; Dyer *et al.*, 1994) have shown a requirement of calcium, and we found PLD activity was stimulated by nonphysiological concentrations range (mM) of calcium (Fig. 5). The calcium concentration for maximal stimulation of PLD activity was about 25 mM. This is much higher than cytosolic concentration of calcium (1-30 μM). It was suggested that this range (mM) of calcium concentration might have biophysical effect such as decreasing surface bilayer fluidity of membranes rather than biochemical effect (Roberts *et al.*, 1986). According to previous report (Yoshida, 1979), membrane-bound PLD could be solubilized by SDS added in the reaction mixture. The effect of SDS on PLD activity is presented in Fig. 6. In the presence of 0.5 mM SDS the PLD activity increased approximately 3-fold. Addition of Triton X-100 in the reaction mixture was found to activate PLD (data not shown). Considering these properties, it seems that the PLD is associated with mitochondrial membranes.

Further studies to determine the substrate specificity of $H_2O_2$-activated PLD were performed. The following 6 classes of mitochondrial membrane phospholipids were examined as possible substrates for PLD: PtdEtn, PtdCho, phosphatidylglycerol, phosphatidylinositol,

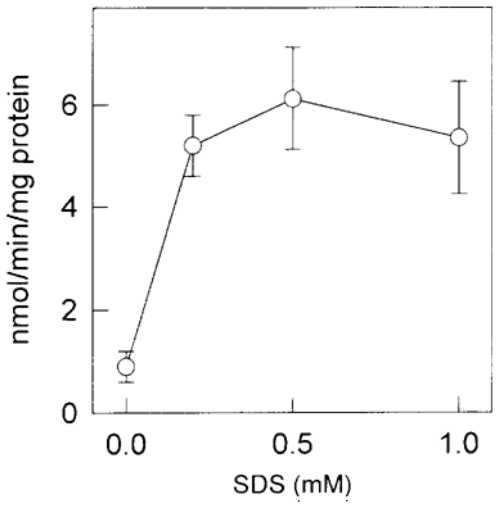


**Fig. 6.** Effects of various concentrations of SDS on mitochondrial PLD activities. Enzyme activities were assayed in 100 mM Mes/NaOH (pH 6.5) buffer for 30 min as described in Materials and Methods. The activity of PLD was measured by release of ethanolamine from phosphatidyl[2-$^{14}$C]ethanolamine. Data represent the mean ±SE ($n$=3).

phosphatidylserine, and cardiolipin. The hydrolyzed product, phosphatidic acid, was measured to determine PLD activity. As shown in Table 1, among many different classes of mitochondrial membrane lipids, PtdEtn and PtdCho were hydrolyzed at a high rate. However, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, and cardiolipin showed only low rate of hydrolysis. These results indicate that $H_2O_2$-activated PLD has PtdEtn as a preferable substrate.

**Table 1.** Substrate specificity of mitochondrial PLD

| Phospholipid substrate | Phospholipase D | |
|---|---|---|
| | Hydrolysis product, PA | Relative activity |
| | nmol mg protein$^{-1}$ min$^{-1}$ | % |
| Phosphatidylethanolamine | 1.75±0.13 | 100 |
| Phosphatidylcholine | 0.60±0.10 | 34.3 |
| Phosphatidylinositol | 0.11±0.04 | 6.2 |
| Phosphatidylserine | 0.17±0.05 | 9.7 |
| Phosphatidylglycerol | 0.26±0.07 | 14.9 |
| Cardiolipin | 0.12±0.13 | 6.9 |

The reaction mixtures consisted of 100 mM Mes/NaOH (pH 6.5), 25 mM $Ca^{2+}$, 50 µg of 100 µM $H_2O_2$-treated mitochondrial protein and each phospholipid substrate. The various substrates were obtained as described in Materials and Methods. The reaction product, PA, was separated by TLC and amount of phosphorus was measured. Relative activity is expressed as percentage of activity for phosphatidylethanolamine. The data represent the mean± SE for four to six experiments. PA=phosphatidic acid.

## DISCUSSION

Recently, our understanding of the role of $H_2O_2$ in plants has been extended. Previous reports showed that infection by pathogens increased the generation of $H_2O_2$ within minute in tomato cell suspension cultures (Vera-Estrella *et al.*, 1992) and $H_2O_2$ had dual effects during chilling imposed oxidative stress on maize seedlings (Prasad *et al.*, 1994). It also suggested that $H_2O_2$ may act as second messenger of salicylic acid in the induction of systemic acquired resistance in plants (Neuenschwander *et al.*, 1995). Thus, it appears that $H_2O_2$ is involved in many aspects of plant metabolism.

Mitochondria are essential cellular components for producing intracellular energy and it has been found that this organelle is a major source of superoxide anion and $H_2O_2$. The main source of mitochondrial $H_2O_2$ is located in the inner mitochondrial membranes (Phung *et al.*, 1994). Therefore, it seems possible that those active oxygen species, which are generated in the mitochondria, could damage the mitochondrial membrane. It is reasonable to assume that $H_2O_2$ might affect the stability of mitochondria and, thereby, normal metabolism of cells.

Under the normal conditions of plant cells, $H_2O_2$ is kept at low steady-state levels by antioxidant systems which include enzymes such as superoxide dismutase, catalase, peroxidase, and nonenzymatic components such as ascorbic acid and glutathione. For example, it has been reported that concentration of $H_2O_2$ was regulated at 0.3-0.9 mM in soybean embryonic axes during germination (Puntarulo *et al.*, 1991). However, high concentration of $H_2O_2$ causes damaging effects in plants; for instance, 50-100 µM of $H_2O_2$ directly inhibits many enzymes (Verniquet *et al.*, 1991). Various environmental perturbations (*e.g.* drought, intensive light, temperature stress) can cause excess active oxygen species. Under such conditions, excess active oxygen species production in plants can result in oxidative damage. It has been known that soybean cells responding to polygalacturonic acid elicitor produce approximately 1.2 mM $H_2O_2$, averaged for mass of cells. (Legendre *et al.*, 1993). It was also reported in mammalian system that $H_2O_2$ is able to reach relatively high (0.1-1.0 mM) local concentration in the presence of activated phagocytes (Morel *et al.*, 1991). Furthermore, it has been suggested that cell components specific oxidative stress leads to organelle specific accumulation

of active oxygen (Willekens *et al.*, 1994).

The results presented here clearly demonstrate that micromolar concentrations of $H_2O_2$ exerts a direct effect on the stability of mitochondrial membranes through loss of membrane protein and phospholipid. The decline of phospholipid amounts seen in our results is consistent with other reports that found a marked decrease in phospholipid content of superoxide anion treated cell membranes (Kendall and McKersie, 1989) or gamma irradiated microsomal membranes (Voisine *et al.*, 1993). Loss of phospholipids is a general feature of membrane deterioration in aging and senescent tissues (Senaratna *et al.*, 1984; Thompson, 1988). Furthermore, the treatment of mitochondria by $H_2O_2$ promotes loss of membrane protein. In our results, the degree of loss of membrane protein upon $H_2O_2$ treatment correlates well with the loss of phospholipids. Therefore, the parameters for membrane degradation, such as depletion of membrane protein and decrease in phospholipid content, are presented here.

There are some indications in the literature that the importance of lipid-degradation enzymes in the membrane deterioration process is suggested during membrane damage by active oxygen. The treatment of 100 μM $H_2O_2$ disrupted the integrity of cell membranes and stimulated the release of arachidonic acid (Boyer *et al.*, 1995). Our data are in agreement with this notion. The results in the present study that exogenous radiolabelled phospholipids were catabolized by mitochondrial protein indicating that enzyme activity may be attributable to a degradation of mitochondrial membranes. Enzymes known to be used for phospholipid degradation are phospholipase $A_1$, phospholipase $A_2$ ($PLA_2$), PLC, PLD, and lipolytic acyl hydrolase. In recent studies, especially $PLA_2$ and PLD are considered to be $H_2O_2$-stimulated phospholipases. Kiss and Anderson (1994) reported that treatment of NIH3T3 fibroblasts with $H_2O_2$ was observed to enhance of PLD mediated hydrolysis of phospholipids. It was also found that $H_2O_2$ stimulated arachidonic acid release from membranes was mediated by a $PLA_2$ and that this process is mediated via kinase activity (Boyer *et al.*, 1995).

Recently, the importance of PLD has emerged in cell signal transduction (Billah, 1993). Many studies suggest that the hydrolysis products of PLD, phosphatidic acid and diacylglycerol, are important intracellular second messengers, and that the activation of PLD occurs through many different mechanisms (Dennis *et al.*, 1991; Harris and Burstein, 1992). However, the role of PLD in signal transduction in plants remains in question. Plant PLD activity was found in cauliflower, tomato, carnation flower, and castor bean leaf (McCormac *et al.*, 1993; Voisine *et al.*, 1993; Dyer *et al.*, 1994). It is not known which specific membrane is associated with PLD. The properties of mitochondrial PLD were very similar to those of early reported plant form of PLD in that it requires a nonphysiological concentration level of calcium and SDS for its optimal assay condition. The mitochondrial PLD has the same substrate specificity as other systems reported, where PtdEtn was a preferred substrate (Harris *et al.*, 1992; McNulty *et al.*, 1992).

The results presented in this report give some insight to the mechanism of membrane degradation by oxidative damage. However, in order to know molecular mechanism of this process, further studies are required such as purification of $H_2O_2$-regulated PLD in mitochondria.

## ACKNOWLEDGEMENTS

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