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Short Communication

Phospholipid Degradation in Frozen Plant Cells Associated with Freezing Injury[1,2]

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ABSTRACT

A striking degradation of phosphatidylcholine into phosphatidic acid was observed in the cortical tissues of less hardy poplar (*Poplus euramericani* cv. gelrica), when the tissues were frozen below a lethal temperature. No change in phospholipids was detected during freezing or even after thawing in the cortical tissues of hardy poplar which survived slow freezing to −30 C or even immersion in liquid N_2 after prefreezing to −50 C. The degradation of phosphatidylcholine during freezing appears to be intimately associated with freezing injury.

There is evidence to show that the primary site of freezing injury in plant cells is the cellular membranes (5, 9, 11). In the electric low frequency resistance studies of alfalfa, Greenham (3) obtained evidence to show that the intactness of the plasma membranes was lost during freezing as a result of injury. In the electrophoretic measurements to study the patterns of freezing in various plant materials, Olien (12) and Sukumaran and Weiser (15) also obtained essentially the same results. These facts suggest that some deleterious changes occur in cellular membranes of plants frozen below the critical temperatures. Thus, it may be postulated that some compositional changes in phospholipids, which are known as the essential components of cellular membranes, occur in frozen plants sustaining injury. In this study with cortical tissues of poplar, phospholipid compositions were determined during freezing, either during or after thawing from various temperatures. Special attention was focussed on the correlation between compositional changes in phospholipids during freezing and injurious effect of freezing on plant cells.

MATERIALS AND METHODS

Materials. As experimental materials, the cortical tissues of poplar (*Poplus euramericana* cv. gelrica) from the campus of our institute were used. The cortical tissues from current twigs of poplar were cut into small pieces (0.5 × 3 cm, about 2 mm in thickness) and then frozen in a test tube (1.3 × 18 cm) at −5 C. After exposure to −5 C for 2 hr, the frozen tissues were cooled in 5 C steps at hourly intervals to succesively lower temperatures to −30 C. They were held at selected test temperatures for 20 hr. Some tissues which were frozen to −30 C were further cooled to −50 C and then immersed in liquid N_2. The frozen tissues were thawed in air at 0 C. After standing at 0 C for 1 hr, they were placed at room temperature for more than 3 days. Freezing injury was then evaluated visually. Browning of the tissues was used as the criterion for rating injury. In some experiments, injury was determined from the extent of release of compounds reactive with $FeCl_3$ from the frozen thawed tissues. For this test, 1 g of the frozen thawed tissue pieces was leached in 5 ml of distilled water at 27 C for 4 hr. Then, 0.1 ml of 10% $FeCl_3$ solution was added to the leaching solution. Dark greenish color developed, depending on the degree of injury. The absorbance was read at 620 nm against the leaching solution from unfrozen samples. The results obtained by this method was found to be comparable with those obtained by visual browning test.

Extraction and Analysis of Phospholipids. Lipid was extracted both from frozen and thawed samples. In the frozen samples, grinding was performed in a cold room at −10 C. To minimize the degradation of phospholipids by phospholipases during grinding, isopropanol cooled at −10 C was added. Isopropanol is known to eliminate lipolytic activity during grinding (7). After grinding, 25 ml of chloroform-methanol (2:1, v/v) cooled at −10 C were added and then lipids were extracted for 1 hr at room temperature. Extraction was repeated twice more with the same solvent, and the combined lipid extracts were subjected to Folch's procedure to remove nonlipid contaminants (2).

Aliquots of the purified lipid samples dissolved in a known amount of chloroform-methanol (2:1, v/v) were loaded on Silica Gel H plates (5 × 20 cm) and were developed with chloroform-methanol-acetic acid (65:25:8, v/v) at 25 C for 50 min. After exposing the plate to iodine vapor, the areas corresponding to each standard phospholipid were scraped into test tubes and directly heated with 0.5 ml of 70% perchloric acid. The lipid phosphorus was determined according to Marinetti's method (10). Nonloaded areas on the same plates were also treated in the same way and used for blanks. Before reading the absorbance, each test tube was centrifuged at 3000 rpm for 3 min to remove silica gel powder.

RESULTS

In the less hardy cortical tissues of poplar collected on October 4, no injury was observed after freezing at −5 C for

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20 hr, whereas they suffered serious injury by freezing below −10 C. As presented in Figure 1, in the cortical tissues frozen below −10 C for 20 hr, a remarkable decrease was observed in phosphatidylcholine, while little or no change was observed in phosphatidylinositol and phosphatidylglycerol. A slight decrease was also observed in phosphatidylethanolamine at −15 C. The decrease in phosphatidylcholine was always accompanied by the inverse increase in phosphatidic acid. The total amount of lipid phosphorous, however, showed no significant change during freezing at any temperature. In the less hardy cortical tissues of poplar from the twigs collected in late September in which the freezing injury increased with the length of time held at −10 C. The time course of the decrease in phosphatidylcholine in the tissues held at −10 C was presented in Figure 2. Phosphatidylcholine decrease with the length of time held at −10 C, which was also accompanied by a concomitant increase in phosphatidic acid. In this experiment also, only a slight change was observed in the other phospholipid components and in the total amount of lipid phosphorus. Thus, the decrease in phosphatidylcholine during freezing seems to be intimately associated with freezing injury of the tissues. To strengthen this notion, some experiments were performed with the hardy poplar cortical tissues collected on November 6. The tissues survived slow freezing to any test temperature down to −30 C or even immersion in liquid N_2 after prefreezing to −50 C. In these frozen tissues, little or no change in phospholipid components was detected. Even after incubation at 27 C for 2 hr following thawing, almost all of the phospholipid components still remained unchanged (Fig. 3). On the other hand, the tissues frozen rapidly by a direct immersion in liquid nitrogen from room temperature (−196RF

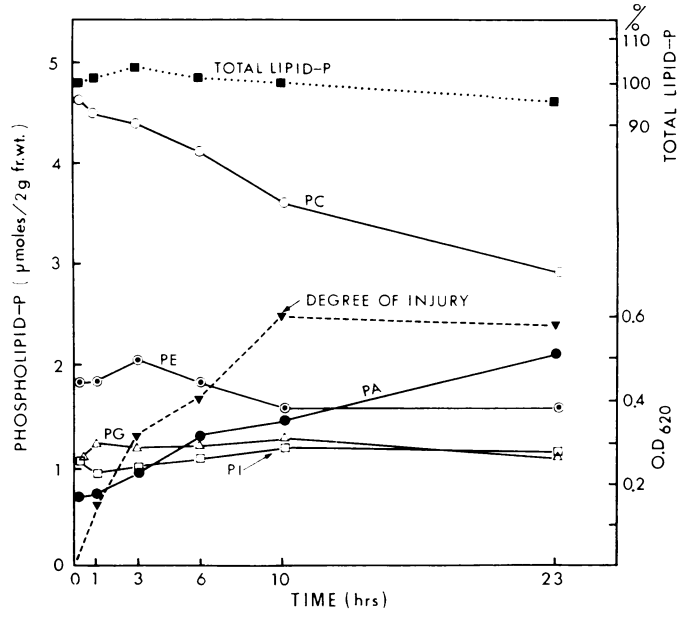

FIG. 2. Time course of decrease in phosphatidylcholine during freezing at −10 C. The tissues were sampled on September 27. They resisted freezing to −5 C, but the injury increased with the length of time held at −10 C. Freezing injury was determined from the extent of release of compounds reactive with ferric chloride from the frozen-thawed tissues and expressed as absorbance at 620 nm. Lipid extractions were performed in the same way as in Fig. 1. The total lipid phosphorus is expressed as the per cent of unfrozen control. Abbreviations are the same as described in Fig. 1.

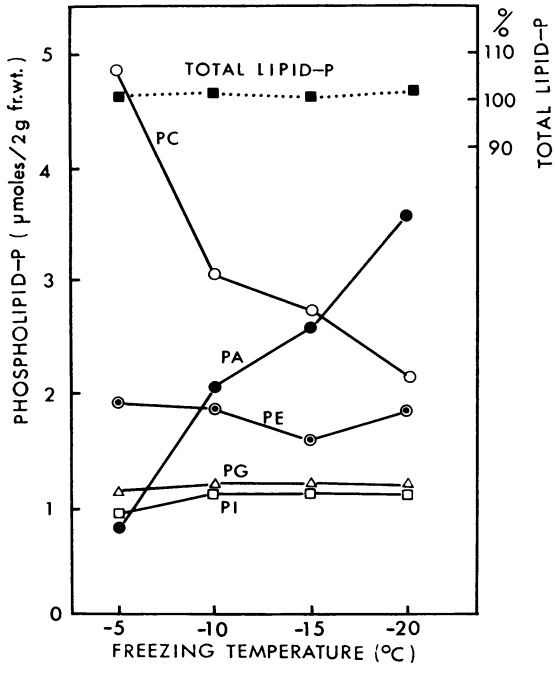

FIG. 1. Changes in phospholipid components and total lipid phosphorus in poplar cortical tissues during freezing. The cortical tissues sampled on October 4 resisted freezing to −5 C. The frozen tissues were ground in a mortar with cooled isopropanol (−10 C) in a cold room at −10 C, then lipids were extracted with chloroform-methanol (2:1, v/v) at room temperature. The amount of total lipid phosphorus is expressed as the per cent of unfrozen control. PC: phosphatidylcholine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PA: phosphatidic acid including a trace amount of diphosphatidylglycerol.

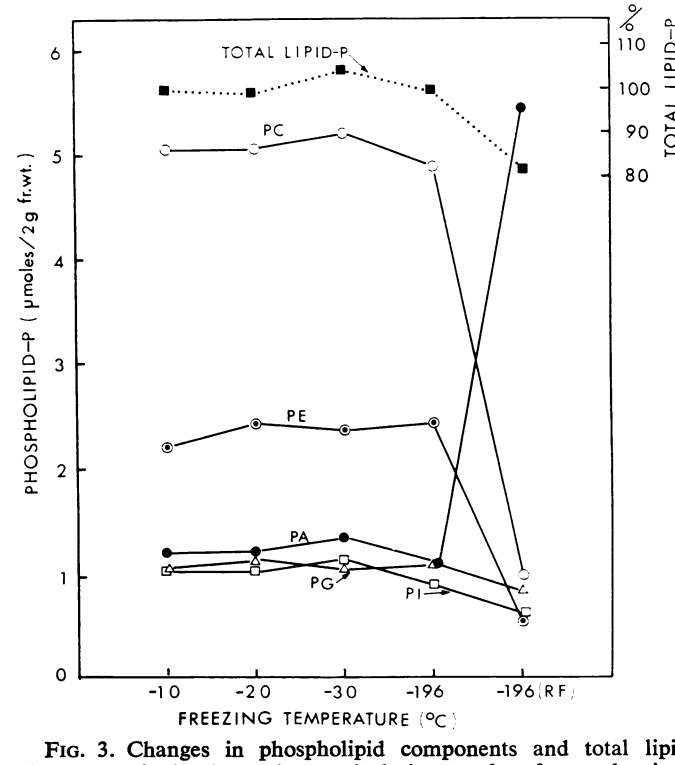

FIG. 3. Changes in phospholipid components and total lipid phosphorus in hardy poplar cortical tissues after freeze thawing. Lipid extractions were all performed after incubation at 27 C for 2 hr following thawing. The amount of lipid phosphorus is expressed as the per cent of unfrozen control. −196: The tissues immersed in liquid N_2 after slow prefreezing to −50 C, which remained alive. −196RF: The tissues frozen rapidly by immersing in liquid N_2 from room temperature, which were seriously damaged.

in Fig. 3) were killed and browning of the tissues was observed immediately after thawing. In these rapidly frozen tissues, however, a drastic change in phospholipid components, including phosphatidylinositol and phosphatidylethanolamine, and a slight decrease in the total amount of lipid phosphorus were observed after thawing.

DISCUSSION

With poplar cortical tissues previously damaged by a direct immersion in liquid N_2 from room temperature, the subfreezing temperatures producing phospholipid degradation were determined by holding frozen tissues at different subfreezing temperatures following removal from liquid N_2. We observed that phospholipid changes proceeded at subfreezing temperatures above −30 C, when the tissues sustained serious injury, but such changes were slowed down below −30 C. On thawing, the changes were drastically accelerated.

We clearly demonstrated that the compositional changes in phospholipids in poplar cortical tissues during freezing or after thawing were always intimately associated with freezing injury. In the leaves of *Aucuba japonica*, we also obtained nearly the same phospholipid degradation during freezing associated with freezing injury, although the degradation patterns in phospholipids differed by species to some degree.

Decrease in phosphatidylcholine in poplar cortical tissues sustaining injury by freezing was always accompanied by a concomitant increase in phosphatidic acid in the tissues. Thus, it seems likely that the decrease in phosphatidylcholine during freezing may be due to an enzymatic reaction catalyzed by phospholipase D which is known to be widely distributed in plants (1, 8, 16, 18). Phospholipase D is also found to be dominantly associated with particulate fractions of poplar cortical cells along with the distribution of phospholipids (S. Yoshida, unpublished data).

In yeast cells sustaining injury by rapid freezing in lipid N_2 or rapid rehydration after freeze-drying, Souzu (14) demonstrated that the amount of total lipids and phospholipids extracted was markedly increased, which was not so in yeast cells cooled or rehydrated slowly. He also observed the marked degradation of phospholipids in rapidly frozen or freeze-dried yeast cells after incubation following thawing or rapid rehydration. He postulated that the rapid removal or addition of water moleclues, which may take part in hydrophobic bonding between lipids and proteins in cellular membranes, may be a dominant factor resulting in a rupturing of the membraneous lipoprotein structure and in subsequent degradation of the phospholipids.

Gusta and Weiser (15, 16) reported that a rapid destruction of nucleic acids by nuclease occurred in plant cells following killing frost and during freeze drying. They postulated from this fact that freezing injury of plants may result from rapid enzymatic degradation of cell constituents following disruption of intracellular compartmentalization, as in animal cells (17, 18). In poplar cortical cells, it may be also postulated that the damage on a specific cellular membrane by freezing, which contributes to the intracellular compartmentalization would cause an activation of phospholipase D and subsequently the degradation of the phospholipids. However, the primary site of freezing injury in cellular membranes, the nature of the deleterious changes induced primarily on cellular membranes by freezing, and the mechanism of the subsequent activation of phospholipase D remain obscure.

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