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Freezing Injury and Phospholipid Degradation *in Vivo* in Woody Plant Cells

I. SUBCELLULAR LOCALIZATION OF PHOSPHOLIPASE D IN LIVING BARK TISSUES OF THE BLACK LOCUST TREE (*ROBINIA PSEUDOACACIA* L.)[1]

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ABSTRACT

The subcellular localization of phospholipase D in homogenates of living bark tissues of the black locust tree (*Robinia pseudoacacia* L.) was examined and found in both soluble and particulate fractions. At least some of the soluble enzyme was considered to be compartmentalized in vacuoles. Considerable amounts of phospholipase D seemed to be tightly bound on several membranes such as endoplasmic reticulum, tonoplast, and a membrane associated with potassium-stimulated ATPase (pH 6.1). The mitochondrial fraction banding at the 40 to 43% (w/w) sucrose layer, however, had the lowest specific activity. The soluble and the particulate phospholipase D were considered to be similar in nature. It is possible that the particulate enzyme, as a part, may be derived from the coexisting nonvesiculated materials visualized in the electron micrograph of each membrane fraction. An involvement of the soluble or the presumed membrane-bound phospholipase D in phospholipid degradation *in vivo* during freezing at sublethal temperatures was discussed with special reference to freezing injury of plant cells.

Phospholipase D is found in the tissues of a wide variety of higher plants (13). Most phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, are enzymically degraded into phosphatidic acid and the related phosphoryl alcohols during extraction of lipid materials with ethyl ether, methanol, and ethanol (15, 22). It has been reported that a striking degradation of phosphatidylcholine into phosphatidic acid occurs in the cortical tissues of less hardy poplar in freezing below sublethal temperatures (23–25 C). The degradation of phospholipids during freezing appeared to be intimately associated with freezing injury. This lipolytic reaction *in vivo* in the frozen state could be detected at a significant rate even at −25 C (24, 25). Similar results have been reported in freezing developing soybean cotyledons (21). In plant cells, phospholipase D is reported to be located both in soluble and particulate cell fractions (2, 3, 7, 18). Only limited data on the cellular location of this enzyme have ever been published (15).

To give some insights into the mechanism of initiating the lipolytic reaction in freezing cells at sublethal temperatures, the subcellular localization of phospholipase D in living bark tissues of black locust tree was examined. A preliminary account of portions of this study has been presented elsewhere (24).

MATERIALS AND METHODS

Plant Materials. The living bark tissues of black locust trees (*Robinia pseudoacacia* L.) sampled in midwinter (hardy tissues) and in early summer (less hardy tissues) was the major experimental material. In some experiments, root tips of corn seedlings (*Zea mays* L., var. "Jubilee") were also used. Seedlings of corn were grown aseptically at 26 C for 3 days in darkness. Root tips, 5 to 10 mm long, were excised with a razor blade, washed, and blotted on filter paper.

Cell-free Extract. The bark tissues were cut into small pieces and ground in a motor-driven mortar and pestle assembly for 2.5 min with washed sea sand at 0 C. The grinding medium consisted of 0.75 M sorbitol, 150 mM Tris-HCl (pH 7.8), 10 mM EGTA, 5 mM DTT, and 1% Ficoll. Five ml of the grinding medium, 0.3 g of Polyclar AT, and 0.6 g of the abrasive were used per g fresh weight of tissues. The brei was squeezed through two layers of gauze and then one layer of Miracloth. Unless stated otherwise, the cell-free extracts were successively centrifuged at 300*g* for 5 min to remove cell debris; 1,500*g* for 10 min, 14,000*g* for 15 min, and 189,000*g* for 60 min were designated as plastids, mitochondria, and microsomes, respectively. Each pellet was washed once with 0.75 M sorbitol-1 mM EGTA-10 mM Tris-HCl (pH 7.6) and resuspended in 6 ml of the same medium as used for washing.

Five g of root tips of corn seedlings were ground in a mortar and pestle in the presence of 2.5 g of washed sea sand and 5 ml of grinding medium (0.5 M sorbitol, 50 mM Tris-HCl [pH 7.6], and 1 mM EDTA [12]). The brei was diluted with 20 ml of the grinding medium and the homogenate was subjected to centrifugation at 500*g* for 10 min. The cell-free extract was then subjected to centrifugation at 20,000*g* for 15 min. The mitochondrial pellet was resuspended in 4 ml of the suspending medium consisted of 0.5 M sorbitol, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.6).

Sucrose Gradient Centrifugation. Six ml of mitochondrial and microsomal suspensions from fresh bark tissues of black locust tree (40 g) were loaded onto 34 ml of a linear sucrose gradient (15–50%, w/w) in 10 mM Tris-HCl (pH 7.6) and 1 mM EGTA, and centrifuged for 2 h at 96,000*g* in a Hitachi SW 27-2 rotor. The discontinuous gradients were prepared by layering in succession 4 ml each of 40, 20, and 15% (w/v) sucrose solution. After centrifugation, 1.2-ml aliquots were collected by using density gradient fractionator, ISCO model 640.

Partial Purification of Soluble Phospholipase D. Winter hardy tissues (70 g fresh weight) of living bark of black locust tree were ground with washed sea sand in grinding medium used for the preparation of cell fractions. The postmicrosomal supernatant was subjected to an ammonium sulfate fractionation (19). The 20% ammonium sulfate precipitate was dispersed in the buffer mixture (0.05 M Tris-HCl [pH 7.6], 1 mM EDTA, 1.5 g 2-mercaptoethanol/l [Tris-EDTA]) and dialyzed overnight against 200 volumes of the same buffer solution. The dialyzed ammonium sulfate fraction was applied to a DEAE-cellulose column (Whatman DE32, 1.8 × 30 cm) previously equilibrated with Tris-EDTA. The column was eluted with 100 ml of Tris-EDTA and then with a linear

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gradient from 300 ml of Tris-EDTA to 300 ml of 1 M KCl in Tris-EDTA. The activity was eluted at 0.3 to 0.55 M KCl. The active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer solution (pH 7.6) containing 2 mM 2-mercaptoethanol.

Enzyme Determination. Antimycin A-insensitive NADH-Cyt *c* reductase was assayed at 25 C by following the reduction of Cyt *c* at 550 nm in the reaction mixture containing 100 μmol of K-phosphate (pH 7.2), 0.5 mg of oxidized Cyt *c*, 5 μmol of NaN_3, 1 nmol of antimycin A, and 50 μl of an enzyme preparation in a final volume of 2 ml. The reaction was started by the addition of 0.5 μmol of NADH.

NADPH-Cyt *c* reductase was assayed in the same reaction mixture as mentioned above but without the addition of antimycin A, and NADPH was substituted for NADH.

Acid phosphatase was assayed in Na-acetate at pH 5.0 with sodium *p*-nitrophenylphosphate as the substrate. The reaction mixture consisted of 200 μmol of Na-acetate (pH 5.0), 10 μmol of substrate, and 50 μl of an enzyme preparation in a final volume of 1 ml. The reaction was performed at 30 C for 7 min, stopped by the addition of 2 ml of 0.1 N NaOH, and then measured at an *A* of 405 nm.

Acid protease was assayed by the method of Beevers (1) with a slight modification. The reaction mixture consisted of 100 μmol of K-phosphate (pH 5.5), 5 mg of casein (Casein nach Hammarstein, Merk), and an aliquot of an enzyme preparation in a final volume of 2 ml. The mixture was incubated at 30 C for 60 min and then terminated by the addition of 0.5 ml of 20% trichloroacetic acid. The sample was centrifuged and the clear supernatant was read at an *A* of 280 nm.

Cyt *c* oxidase was assayed spectrophotometrically at 25 C by following the oxidation of Cyt *c* at 550 nm. The reaction mixture contained 100 μmol of K-phosphate (pH 7.2), 0.42 mg of Cyt *c* reduced with Na-dithionite, 0.1% Triton X-100, 1 μM EDTA, and 20 μl of an enzyme preparation in a final volume of 2 ml.

ATPase was assayed by the method described by Hodges *et al.* (5, 16). The reaction mixture contained 3 μmol of ATP, 1.5 μmol of $MgCl_2$, 50 μmol of KCl, 100 μmol of Tris-Mes (pH 6.1) or Tris-HCl (pH 7.8), and 0.2 ml of an enzyme preparation in a final volume of 1 ml. The reaction was carried out at 30 C for 30 min and then stopped by the addition of 1 ml of 10% trichloroacetic acid. One drop of 1% BSA solution was added immediately before the termination of the reaction to make the supernatant clear. The released Pi was determined by the Fiske-SubbaRow method (4).

Assay of phospholipase D using added substrate was carried out in the reaction mixture containing 3 μmol of sonicated egg phosphatidylcholine purified through silicic acid column chromatography, 1.5 μmol of SDS as the activator (19), 60 μmol of Na-acetate (pH 5.5), 50 μmol of $CaCl_2$, and 0.2 ml of an enzyme preparation in a final volume of 1 ml. Preliminary experiments showed that membrane-bound phospholipase D could be solubilized by SDS added in the reaction mixture. The reaction was performed at 30 C for 30 min and then terminated by the addition of 0.2 ml of 1 N $HClO_4$ solution. One drop of 1% BSA solution was added immediately before the termination of the reaction. The samples were frozen at −20 C for 2 h before centrifugation. The liberated free choline in the supernatant was determined by the iodine-potassium iodide method (8).

Protein was determined by the method of Lowry after precipitating with trichloroacetic acid (10). BSA was used as the standard.

Electron Microscopy. Membrane pellets obtained from a linear sucrose gradient centrifugation were resuspended in 2.5% glutaraldehyde in phosphate buffer and fixed for 3 h at 0 C. The pellets were rinsed through subsequent changes of phosphate buffer solution. Postfixation was done in 2% buffered osmium tetroxide solution and dehydrated in increasing percentages of ethanol and *n*-butylglycidylether. The dehydrated pellets were embedded in Spurr's epoxy resin (17). The section were stained with saturated uranylacetate and Reynold's lead solution (14) and viewed with a JEM 100 C electron microscope.

RESULTS

Phospholipase D in living bark cells of the black locust tree was found both in particulate and soluble cell fractions after differential centrifugation (Table I). There were no significant differences in total activity *in vitro* between the supernatant from summer less hardy tissues and winter hardy tissues. Total activities in particulate fractions, however, declined significantly from winter to summer. Although the highest percentage of the total activity was distributed in soluble fractions, the highest specific activity was associated with the microsomal fraction in hardy tissues and with the plastid fraction in summer tissues.

To get more detailed information concerning the distribution of phospholipase D in various membranes, pellets of both 14,000 and 189,000*g* were separated on a linear sucrose gradient. Peaks of antimycin A-insensitive Cyt *c* reductase occurred in fractions 6 (at the interface between sample and 15% sucrose), 11 (23% sucrose), and 25 (41% sucrose) in the 14,000*g* pellet, and fractions 6 (at the interface between sample and 15% sucrose) and 10 (22% sucrose) in the 189,000*g* pellet (Fig. 1). Based on the enzyme

Table I. *Subcellular Localization of Phospholipase D in Cells from Winter Hardy and Summer Less Hardy Tissues*

Protein and total activity were expressed on the basis of 10 g of fresh material. Activity was assayed in *in vitro* system as described in the text.

Cell fraction	Protein		Total Activity		Specific Activity	
	Hardy	Less hardy	Hardy	Less hardy	Hardy	Less hardy
	mg		*μmol/h*		*μmol/mg·h*	
Plastids (300–1,000*g*)	11.5	2.2	62.0	25.3	5.4	11.5
Mitochondria (1,000–14,000*g*)	13.6	4.2	85.7	26.9	6.3	6.4
Microsome (14,000–189,000*g*)	10.8	4.6	97.8	25.3	9.0	5.5
Supernatant	103.8	37.2	172.9	155.6	1.7	4.2
Total	139.7	48.2	418.4	233.2		

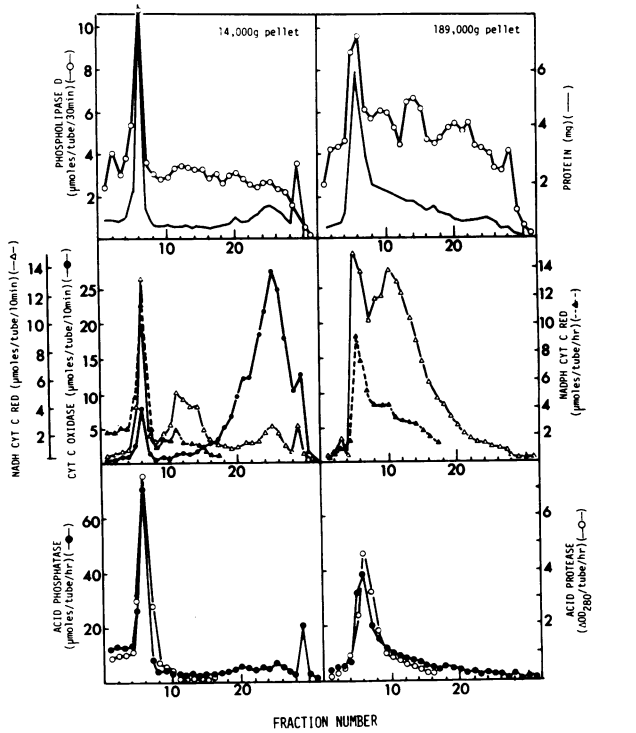

FIG. 1. Fractionations of enzymes in 14,000*g* and 189,000*g* pellets by linear sucrose gradient centrifugations. Forty g of winter hardy tissues were used in this experiment.

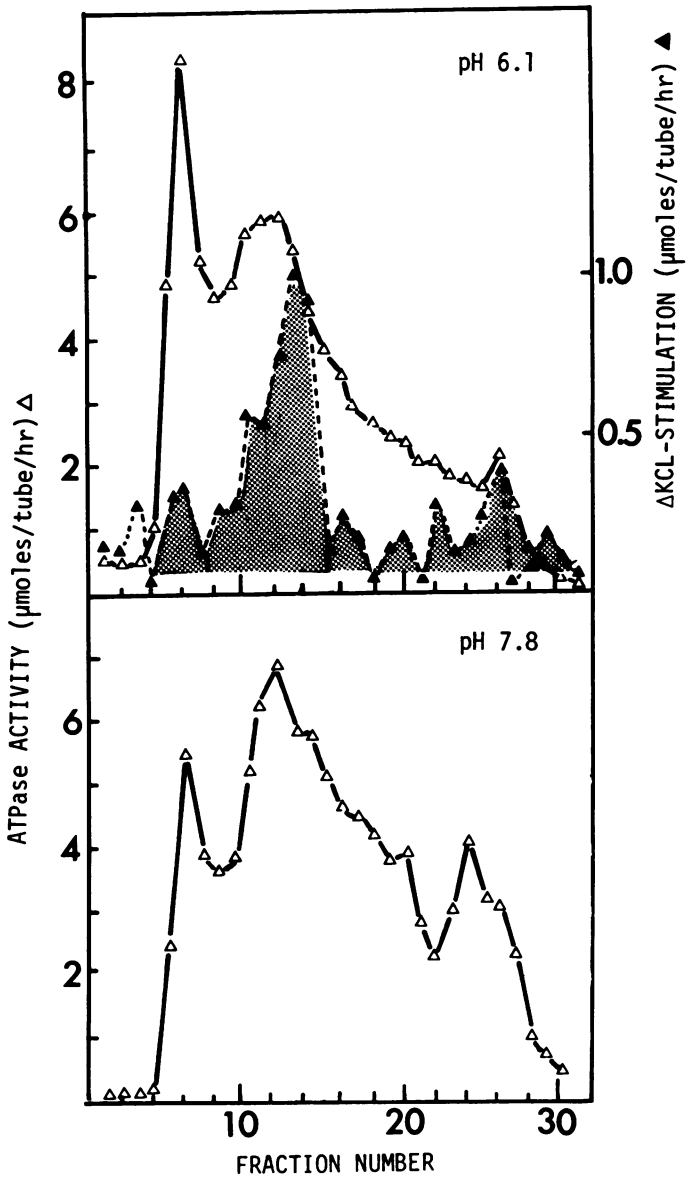

FIG. 2. Distribution profiles of ATPase in microsomal pellet from winter hardy tissues after linear sucrose gradient centrifugation. Activities were assayed in the presence of 1.5 mM $MgCl_2$ and 50 mM KCl at different pH values. Shaded area corresponds to stimulation by KCl.

distribution and the apparent density (nearly 1.089 g/cm^3), fraction 10 in the 189,000*g* pellet was identified as smooth ER. NADPH-Cyt *c* reductase was specifically located in fraction 6 in both the 14,000 and 189,000*g* pellets. There seems to be a definite difference in the distribution profiles of both Cyt *c* reductases. Acid hydrolases, such as phosphatase and protease, were concentrated in fraction 6 in both the 14,000 and 189,000*g* pellets. From the distribution of Cyt *c* oxidase, it can be seen that mitochondria banded in fractions 24 to 26 (40–43% sucrose).

ATPase activity profiles of the 189,000*g* pellet on a linear sucrose gradient centrifugation are shown in Figure 2. At pH 6.1, two peaks of activity occurred at fractions 6 and 12. KCl-stimulated ATPase activity at pH 6.1 was associated with fractions 10 to 14 (22–27% sucrose). At pH 7.8, three overlapping peaks of ATPase occurred at fractions 6, 12, and 24. Taking account of a high concentration of acid phosphatase in fraction 6, the acid ATPase (pH 6.1) at fraction 6 may be partly due to acid hydrolase activity. However, even at alkaline pH, still relatively high activity of ATPase was detected in this fraction. Accordingly, some kinds of ATPases are likely to be distributed in this fraction. Recent studies indicate the presence of ATPases on tonoplast-like smooth membranes (9, 16) and on plasmalemma membranes (5, 6).

In the 14,000*g* pellet, the highest activity of phospholipase D occurred at fraction 6 which also had Cyt *c* reductase (Fig. 1). Other activity was also distributed over heavier membrane fractions. No distinct peak was determined at fractions 24 to 26 where mitochondria were banded. In the 189,000*g* pellet, possible peaks of phospholipase D were observed at fractions 6, 9, 13, 20, and 28 (Fig. 1). Cyt *c* reductase and phospholipase D coincided in fractions 6 and 9. A peak of phospholipase D at fraction 13 coincided with the peak of potassium stimulated ATPase (pH 6.1).

When the pelleted bands from fraction 6, both from 14,000 and 189,000*g* pellets, were examined by electron microscopy, it was found to be composed of a heterodisperse mixture of small vesicles (Fig. 3.1 and 3.3). An electron micrograph of fraction 24 from the 14,000*g* pellet, where the peak of Cyt *c* oxidase occurred, revealed the presence of morphologically intact mitochondria (Fig. 3.2). An electron micrograph of fraction 10 from the 189,000*g* pellet showed the presence of tiny vesicles (Fig. 3.4).

Table II shows the effect of hypotonic treatment and additional freeze-thawing in liquid N_2 on release of protein, acid phosphatase, and phospholipase D from various fractions separated on sucrose gradient centrifugation. In the preliminary experiment, I observed that subjecting 14,000 and 189,000*g* pellets to a hypotonic treatment, *i.e.* suspension in 5 mM Tris-HCl (pH 7.6) containing 1 mM EGTA, resulted in a marked reduction both in the packed volumes and the turbidity, releasing protein and enzymes into the supernatant. In the present experiment, freeze-thawing was additionally utilized to ensure the breakage of vesicle-entrapped soluble materials. In the 14,000*g* pellet, remarkable reductions in all three were observed both in fractions 6 and 24 to 26 after the treatments. In the 189,000*g* pellet, a leakage of phospholipase D from fraction 6 after the treatments was observed to be relatively small as compared with the leakage of protein and acid phosphatase. Only minor changes were observed in phospholipase D activity due to the treatments in the other fractions, except for fractions 18 to 20. When rerun on sucrose gradients after hypotonic treatment and subsequent freeze-thawing, presumed ER membrane vesicles (fractions 9–11) retained both phospholipase D and NADH-Cyt *c* reductase (Fig. 4).

To check the possibility of nonspecific binding of soluble phospholipase D on several membranes during the homogenizing process, new membrane materials, *i.e.* thylakoid membranes (10.0 mg protein) from spinach leaves, and mitochondrial (12.8 mg protein) and microsomal (10.1 mg protein) membranes from potato tubers, respectively, were mixed with a sufficient amount of the soluble supernatant (76 mg protein), containing soluble phospholipase D (total activity; 106 μmol/h), from hardy bark cells of the black locust tree. After incubation for 30 min at 0 C, the membrane materials were pelleted from the supernatant by centrifugation and phospholipase D activity was assayed *in vitro* with added substrate as described in the text. There was no indication of nonspecific adsorption of the soluble enzyme to the new membranes during the mixing process (data not shown).

Further experiments were carried out to isolate heavy and light lysosomal apparatus from 20,000*g* pellets of root tip cells of corn seedlings after a discontinuous sucrose gradient by the method of Matile (12). Distinct peaks of acid phosphatase, acid protease, and acid nuclease (data not shown) and NAD(P)H-Cyt *c* reductase were determined at the interface between sample and 15% sucrose, and the interface between 20 and 40% sucrose layers, as reported originally by Matile (12). These two fractions correspond to lighter and heavier lysosomal organelles, respectively, and contain phospholipase D as well (Fig. 5). Thus, soluble phospholipase D is also one of the lytic enzymes contained in vacuoles and provacuoles in root tip cells of corn seedlings. When these fractions containing acid hydrolases and phospholipase D were subjected to a hypotonic treatment, a considerable part of phospholipase D as well as the other hydrolytic enzymes leaked into the supernatant.

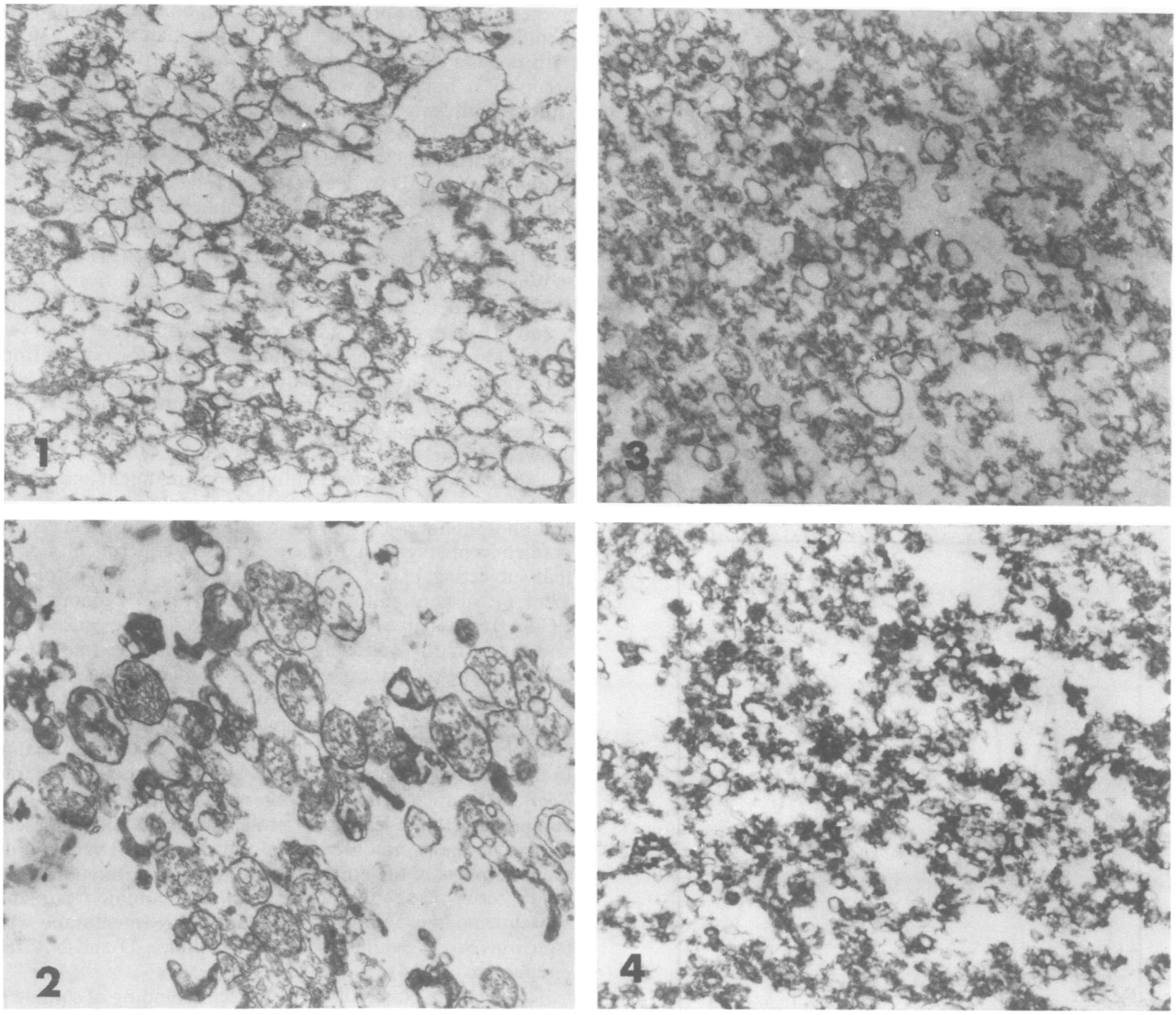

FIG. 3. Electron micrographs of pooled fractions obtained by sucrose gradient centrifugation. 1 and 2: Fractions 6 and 24 from 14,000*g* pellet; 3 and 4: fractions 6 and 10 from 189,000*g* pellet (× 10,000).

Table II. *Effect of Hypotonic Treatment and Additional Freeze-Thawing (HF) on Enzyme Activities in Vitro in Subfractions from Sucrose Gradient Centrifugations*

Winter hardy tissues were used as the experimental material. Each subfraction was diluted by 8 volumes of 5 mM Tris-HCl containing 1 mM EGTA (pH 7.6) and subsequently subjected to freeze-thawing in liquid nitrogen. After centrifugations, protein and enzymes retained in the pellets were assayed. The experimental details are described in the text. The subfraction numbers refer to Figure 1.

Subfractions	Tentative Identification	Protein			Acid Phosphatase			Phospholipase D		
		Control	HF	% Retained	Control	HF	% Retained	Control	HF	% Retained
		mg			*μmol/fr·h*			*μmol/fr·h*		
14,000*g* pellet										
Fr 5–7	Tonoplast vesicles	7.7	2.9	37.6	522	144	25.7	76.4	30.8	40.3
Fr 24–26	Mitochondria	5.1	3.6	70.6	246	100	40.6	43.6	19.6	44.9
189,000*g* pellet										
Fr 5–7	Tonoplast vesicles	10.1	5.1	50.5	624	308	49.3	66.7	55.1	82.6
Fr 9–11	Smooth ER	3.4	2.6	76.4	243	154	63.3	53.9	55.4	102.7
Fr 12–14	Membranes associated with K-stimulated ATPase	2.8	2.7	96.4	140	114	81.4	53.2	47.9	88.8
Fr 18–20		1.8	1.6	88.9	85	43	50.5	49.2	22.6	45.9
Fr 21–23		2.0	2.0	100	80	52	65.0	29.1	20.5	70.4

Soluble phospholipase D from bark tissues of black locust tree was first precipitated by 20% saturated ammonium sulfate and then purified on a DEAE-cellulose column. The majority of activity was eluted at KCl concentration between 0.3 and 0.55 M, as reported for soluble phospholipase D from peanut seeds (19). The partially purified soluble phospholipase D and membrane-

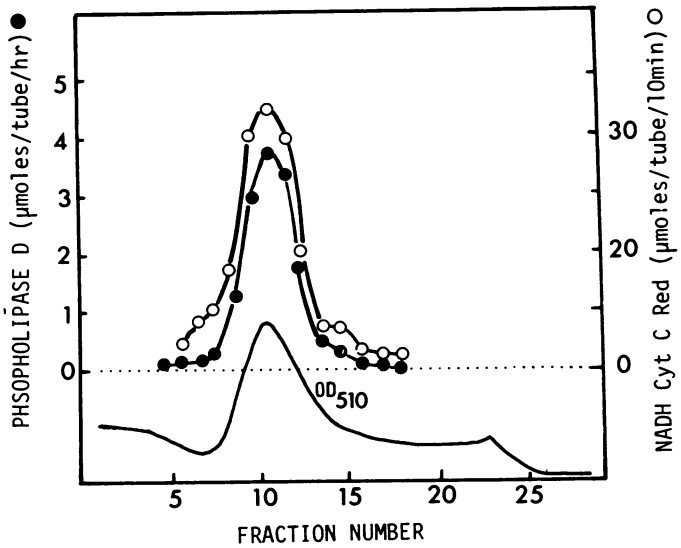

FIG. 4. Distribution patterns of phospholipase D and antimycin A-insensitive NADH-Cyt *c* reductase in smooth ER. Fractions 9 to 11, obtained by linear sucrose gradient centrifugation of 189,000*g* pellet (Fig. 1), were diluted by 5 mM Tris-HCl (pH 7.6) contained 1 mM EGTA, were subsequently freeze-thawed in liquid N_2, and recentrifuged on linear sucrose gradient. Turbidity at 510 nm (——); phospholipase D (●); NADH-Cyt *c* reductase (O).

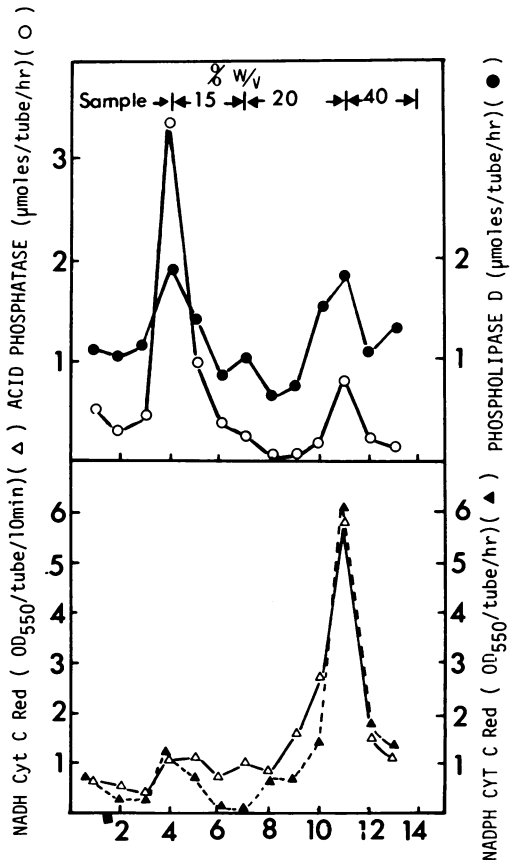

FIG. 5. Fractionation of enzymes in 20,000*g* pellet of cell-free extract from corn root tip cells by discontinuous sucrose gradient centrifugation after the procedure of Matile (12).

bound phospholipase D (smooth ER as the enzymic source) gave the same pH activity profile in an *in vitro* reaction system, having pH optimum at 5.5 in Tris-maleate buffer (data not shown). Based on the similarity in the pH activity profile, soluble and membrane-bound phospholipase D may be the same enzyme in nature.

DISCUSSION

The results of this study suggest that phospholipase D exists as soluble and membrane-bound forms in bark cells of black locust tree. Similar results have been reported with cellular distribution of phospholipase D in spinach leaves (15).

After washing with 5 mM Tris-HCl (pH 7.6) containing 1 mM EGTA, the 189,000*g* pellet contained no detectable amount of soluble phospholipase D. Neither washing with KCl solution ranging from 0.1 to 0.5 M nor freeze-thawing in liquid N_2 could release the enzyme from the EGTA-washed pellets, whereas considerable amount of activity was recovered in the soluble fraction after extracting the pellet with 3.5 mM SDS in 5 mM Tris-HCl (pH 7.6) (data not shown). When membrane materials from other plants with a relatively low content of phospholipase D, such as thylakoid membranes from spinach leaves, mitochondrial and microsomal membranes from potato tubers, were mixed with the sufficient amount of soluble supernatant from hardy bark cells of the black locust tree, there was no indication of nonspecific adsorption to the new membranes. Thus, the possibility of nonspecific binding of phospholipase D on membranes during the homogenizing process can be ruled out. The content of phenolics in the bark tissues of black locust tree is much less than those of the other woody species such as willows and poplars. We have carefully checked the effective amount of Polyclar AT to be used for neutralizing phenolics in the bark tissues. When more than 0.2 g of Polyclar AT was utilized per g of the bark tissues, a negligible amount of phenolics was detected in the homogenates and no contamination of phenolics was observed in the pellets. In the present study, 0.3 g of Polyclar AT was utilized per g of the tissues. Accordingly, the disturbing problems of phenolics in isolating membrane materials were overcome. Thus, it seems very likely that a significant amount of phospholipase D is tightly bound on various membranes such as ER, tonoplast, and other unidentified components (Fig. 2 and Table II). Relatively low specific activity, however, was found in the mitochondrial fraction. Some uncertainty remains due to the presence of nonvesiculated, apparently precipitated materials, to be seen together with vesicles in the fractions 6 of gradient profiles of both the 14,000 and 189,000*g* pellets (Fig. 3.1 and 3.3). Similar materials can be observed in the vacuoles of intact cells of the wintering bark tissues of these trees (data not shown). Further work is needed to identify the nature of the particles accompanying vesicular membranes in the sucrose gradient. If it turns out that they are identical to those observed in vacuoles it is not likely that they will be the source of bound phospholipase D measured in these studies.

Fraction 6 from sucrose gradients of both 14,000 and 189,000*g* pellets was tentatively considered to be vesicles derived from tonoplast membranes, formed by rupturing and resealing during mechanical grinding. NADPH-Cyt *c* reductase was specifically located in these fractions. The Cyt *c* reductase has been reported to be closely associated with tonoplast-like membranes in cells of *Catharanthus roseus* (11). Thus, the protein and enzyme rendered soluble from this fraction by a hypotonic treatment and subsequent freeze-thawing in liquid N_2 were probably soluble materials from the vacuoles and/or cytosol, included during the vesiculation process. If so, I speculate that a considerable part of the phospholipase D of black locust bark cells might be soluble, and compartmentalized in vacuoles. According to the sucrose gradient of the 20,000*g* pellet from root tip cells of corn seedlings, soluble phospholipase D is considered to be one of the lytic enzymes in the lighter and heavier lysosomal apparatus (Fig. 5). Recently, direct evidence showing the presence of soluble phospholipase D in plant vacuoles was obtained in our laboratory by using intact vacuoles isolated from protoplasts of carrot roots (Yoshida, un-

published data). Waldale and Galliard (20) have reported that lipid-degrading enzymes, lipolytic acylhydrolase, and lipoxygenase are localized in the "lysosomal" fractions of cabbage, potato, and pea.

Freezing of less hardy plant cells could result in either disturbance of the compartmentation of soluble phospholipase D or loss of a regulatory properties of the membrane-bound enzyme. As a result, a drastic degradation of membrane phospholipids *in vivo* may proceed even in the frozen state in association with freezing injury of plant cells.

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