Enzymic and Protein Character of Tonoplast from Hippeastrum Vacuoles

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ABSTRACT

The membrane of anthocyanin containing Hippeastrum petal vacuoles was examined for protein and enzyme content after purification by equilibrium density centrifugation. Light scattering, protein, and a Mg2+-dependent nucleotide-specific ATPase were associated with membrane having a density of 1.08 to 1.12 grams per cubic centimeter. A small amount of acid phosphatase was also present in this region of the gradient, but this activity peaked at about 1.12 grams per cubic centimeter. A component of yeast tonoplast, α-mannosidase, was not significantly present. UDP-glucose, anthocyanidin-3-O-glucosyltransferase, thought to be a cysteine enzyme in Hippeastrum, was absent from tonoplast of vacuoles isolated by osmotic shock in 0.2 molar K2HPO4 or 0.35 molar mannitol. Vascular acid phosphatase was insensitive to ethylenediaminetetraacetate but was 80% inhibited by 10 millimolar KF, while ATPase was inactivated by 2 millimolar ethylenediaminetetraacetate and only 50% inhibited by 10 millimolar KF. Five major and about 9 minor polypeptides were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane protein on 5 to 30 and 6 to 16% gradient gels.

The osmotic, storage, and sequestration functions of the mature plant vacuole undoubtedly require tonoplast proteins for facilitating active transport between the vacuolar compartment and the cytosol. Proton (16) and sugar (9, 10) transport into vacuoles recently has been demonstrated, using isolated vacuoles. Cyclization in transvacuolar strands, the formation and maintenance of transvacuolar strands, and the uptake processes in vacuoles may involve tonoplast-associated microfilaments. As studies of transport and other functions of the vacuole progress, a better understanding of the chemical character of the tonoplast will be required. This work describes characterization of the enzymic and protein makeup of tonoplast obtained from vacuoles isolated by K2HPO4-induced osmotic shock of pigmented Hippeastrum petal protoplasts.

MATERIALS AND METHODS

Protoplasts and vacuoles of Hippeastrum petals (cv. Dutch Red Hybrid) and other tissues were prepared as previously described (25). Washed vacuoles were lysed with 2% w/w sucrose; 10 mM HEPES, NaOH (pH 8); 3 mM MgSO4; 0.5 mM DTT and were centrifuged at 100,000g for 2 h at 4 °C. The pellet was resuspended in the above solution and applied to a 15 to 50% w/w linear sucrose gradient (60% w/w sucrose cushion). The gradient contained buffer, MgSO4, and DTT as above. Sucrose (grade 1) was obtained from Sigma. Gradients were centrifuged at 100,000g for 17 h at 4 °C or 20 °C. Those membrane samples which were sonicated were treated for 30 s at 0 °C using the 3 setting of a sonifier cell disrupter-microtip (Heat Systems-Ultrasonics, Inc., Plainview, NY). Protein was measured by the method of Bradford (5). Light scattering was monitored as A560. Densities were determined at 20 °C by refractive index measurement. PNPPase, α-mannosidase, and phosphodiesterase were assayed at pH 5.5 in 0.125 mM Na nitrate using 6 mM substrate (6). ATPase (17) and UDP-glucose, anthocyanin-3-O-glucosyl transferase (12) were assayed as previously described.

Polypeptide profiles were obtained by SDS-PAGE on 2-mm-thick slab gels (5 to 30% acrylamide) using the apparatus described by Studier (22) or 3-mm i.d. tube gels (6 to 16% acrylamide). For both, the gel and buffer system of Laemmli (15) was used. Membrane (density 1.04 to 1.12 g per cubic centimeter) was recovered from gradients, diluted with vacuole lysing medium (above), and recovered by centrifugation at 100,000g using 3-mi-thick walled polyacrylamide tubes (Beckman). Pellets were prepared for electrophoresis by boiling (5 min) with 100 µl 2% SDS, 10% glycerol, 1 mM EDTA, 10 mM K-phosphate (pH 8), 40 mM DTT, after which residual insoluble material was removed by centrifugation at 100,000g for 10 min at 25 °C. Protoplasts were washed 3 consecutive times with 0.7 M mannitol; 20 mM Mes, KOH (pH 5.5) (protoplast to wash: 1:160, v/v) and finally sedimented at 800g. Protoplast lysate, enriched cytosol (25), and vacuole sap fractions were prepared for electrophoresis in the same manner as that described for tonoplast. Samples were separated by electrophoresis at room temperature and 220 mamp h (maximum current 20 mamp) for 5 to 30% slab gels and 2 mamp/tube for 10 h for 6 to 16% tube gels. CF3, prepared according to Strotmann et al. (21), Cellulysin, and T7 phage protein were similarly treated. After electrophoresis, gels were stained with Coomassie blue and destained essentially as described by Laemmli (15). PAS staining for glycoprotein was according to Zacharius et al. (27). For tube gels, select bore glass tubing (Bio-Rad) was used. Gels were removed by breaking the tubes with a gel tube eliminator (Bio-Rad). Tube gels were scanned at 600 nm for Coomassie blue and at 550 nm for PAS stain. Lactoperoxidase-catalyzed iodination was performed according to Morrison (13), using carrier free 125I-NaI. Purified membrane recovered from approximately 9 x 109 vacuoles was labeled using 1 mCi of 125I-NaI, using 5 additions of H2O2 after which the reaction was stopped with Na2S and NaI and centrifuged at 100,000g for 1 h at 4 °C. The pellet was washed (by resuspension and sedimentation) 5 times with 70 µM Na2SO4 to remove 125I-NaI. For delipidation, labeled membrane was extracted with CHCl3:methanol, 2:1, and the extract centrifuged at 100,000g for 2 h at 4 °C. Protein was treated with SDS and electrophoresed, as described, after which gels were sliced into 1-mm discs and

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2 Abbreviations: PNPP, P-nitrophenylphosphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; CF3, spinach chloroplast coupling factor; PAS, periodic acid Schiff.

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counted directly. Molecular weights were determined by extrapolation from a plot of log mol wt versus $R_F$ of the 150, 86, 83, 62, and 38 kdalton polypeptides of T7 phage (22).

**RESULTS**

Density and Enzymic Contents. Purification of tonoplast on 15 to 50% sucrose gradients resulted in two peaks of protein and light scattering (membrane), a small peak centered at density 1.01, and a major peak with a density 1.08 to 1.12 g per cubic centimeter (Fig. 1). Similar results were found for *Tulipa* petal and leaf tonoplast (not shown). Both PNPPase (6) and Mg$^{2+}$-dependent ATPase (17) have been shown to be associated with *Hippeastrum* vacuoles, the latter with partially purified membrane. Analysis here indicated a clear association of Mg-ATPase activity with gradient purified tonoplast (Fig. 2). The yeast tonoplast marker (24), $\alpha$-mannosidase, was not clearly present. The levels of this activity were near the minimum level of detection monitoring PNP released from $p$-nitrophenyl-$\alpha$-D-mannoside. The level of PNPPase entering the gradient was a very small fraction of that present in the sap of vacuoles used to prepare tonoplast applied to the gradient. PNPPase was barely detected by monitoring Pi but was clearly found to be present when released PNP was measured. Detection of PNP was found to be 30 times more sensitive than Pi detection, using the methods described. PNPPase activity peaked at about 1.12 g per cubic centimeter, a somewhat higher density than the main ATPase peak at 1.1 g per cubic centimeter.

Sonication of vacuoles to prepare tonoplast resulted in a sharpening of the ATPase peak at 1.1 g per cubic centimeter and a decrease in the low density peak (Fig. 3). This treatment also caused an increase in PNPPase in both peaks, suggesting that this activity may be, at least in part, associated with isolated tonoplast as a result of membrane preparation.

The nucleotide specificity of partially purified tonoplast was determined. Vacuoles were isolated, washed, and then lysed with 20 mM Hepes (pH 8), 2 mM DTT, 0.3 mM MgCl$_2$. The resulting solution was applied to a step gradient consisting of 20 and 38% sucrose (w/w) steps each containing buffer, DTT, and MgCl$_2$, as in the lysing medium. Gradients were centrifuged at 150,000g for 45 min at 15°C. Membrane was recovered from the 20/38% sucrose interface and tested for phosphohydrolase activity using ATP, ADP, AMP, CTP, GTP, UTP, and PNPP as substrates at 3 mM. Activities relative to ATPase (100%) were 6, 2, 15, 66, 30, and 1.5%, respectively.

The possibility that UDPG, anthocyanidin-$3-O$-glucosyl transferase might be tonoplast bound was tested using purified and unpurified (pellet prior to gradient purification) tonoplast. Transferase activity was not detected in either preparation. Unpurified tonoplast, obtained from vacuoles prepared by gentle osmotic shock in both 0.2 M K-phosphate, (pH 8) and 0.35 M mannitol, 30 mM Hepes/NaOH (pH 8) lacked activity. Enriched cytosol (25) formed anthocyanin, as observed earlier (12), and also hydrolyzed UDP-glucose to form free glucose. Tonoplast did not form glucose from UDP-glucose.

**KF Inhibition of PNPPase and ATPase.** The sensitivity of *Hippeastrum* vacuolar ATPase and PNPPase to EDTA and KF were examined. As shown in Figure 4, PNPPase was insensitive to EDTA but was inhibited by 80% in the presence of 10 mM KF. Mg-ATPase was inactive in the presence of 2 mM EDTA but was...
inhibited 40 to 50% by 10 mm KF. Similar results were obtained for PNPPase of enriched cytosol of *Hippeastrum* and phosphodiesterase (bis-PNPPase) of vacuolar sap.

**Protein Character of Tonooplast.** The polypeptide profile of gradient-purified tonoplast, as determined by SDS-PAGE on a 5 to 30% slab gel, is shown in Figure 5A. Marker peptides from T7 phage and subunit peptides of CF₁ are also shown. Tonooplast preparations contained 5 major peptides (labeled bands 3, 6, 7, 8 and 9) having mol wts of 69, 59, 57.5, 55, and 53 kdaltons, respectively. Nine minor polypeptides were also discernible on the gels. The mol wts for the α and β subunits of CF₁ were calculated to be 60 and 56 kdaltons, respectively, as compared to literature values of 59 and 56 kdaltons (2). Some differences, and also similarities, were observed when banding patterns of vacuole sap, enriched cytosol, prooplast lysate, isolation enzyme, and unpurified tonoplast (all shown in Fig. 5B) were compared with purified tonoplast (Fig. 5A). Cytosol and prooplast samples contained many polypeptides which were not observed in the vacuole sap and tonoplast samples. Unpurified tonoplast contained one major band (about 76 kdaltons) which was absent from purified tonoplast. The main polypeptides of tonoplast (bands 3, 6, 9; Fig. 5A) were absent from vacuole sap. Analysis of purified tonoplast on 6 to 16% gradient tube gels and subsequent scanning of staining patterns produced the profile in Figure 6A. The major peptides identified as 1 to 14 corresponded in calculated molecular weight to those determined from slab gels (Fig. 5). PAS staining suggested that the major, and at least most minor, peptides contained little or no carbohydrate (Fig. 6A). Presence of substantial PAS stain at the front suggested the possible presence of glycolipid. Labeling of membrane with 125I by lactoperoxidase-catalyzed iodination resulted in the labeling of the major peptides and substantial labeling of material moving with the front (Fig. 6B). These results are consistent with the labeling of lipid. 125I patterns of major membrane peptides were qualitatively similar to Coomassie blue patterns, except for the presence of lactoperoxidase in the former. Lactoperoxidase (about 90 kdaltons), which was labeled, was not totally removed from membrane during the washing of membrane to remove 125I-NaI. There were qualitative differences between Coomassie blue and 125I patterns. Such differences are often observed and are undoubtedly due to the dependence of lactoperoxidase labeling on tyrosine content. Removal of lipids from 125I labeled membrane resulted in a relative decrease in the amount of label moving with the front, suggesting the association of 125I with lipids rather than with small peptides (Fig. 6B).

**DISCUSSION**

The results of this study indicate that tonoplast of pigmented *Hippeastrum* petal cells has a density of 1.08 to 1.12 g per cubic centimeter and bears a Mg-dependent, nucleotide specific, ATPase activity. These observations are consistent with the results of others regarding the density of this membrane (4, 11, 19) and substantiate earlier observations which indicated that *Hippeastrum* vacuole ATPase was tonoplast bound (17). Lin et al. (17) made no
claims that the ATPase described for *Tulipa* and *Hippeastrum* vacuoles would serve as a tonoplast marker. Indeed, it was pointed out that several similarities existed between vacuole ATPase and both plasma membrane and mitochondrial ATPase. The suggestion that Lin et al. (17) regard vacuole ATPase as a tonoplast marker was incorrectly inferred and referenced (19).

Acid phosphatase, while present in large amounts in the vacuolar sap of *Hippeastrum* (6), was not substantially associated with purified tonoplast. Further, the method of membrane preparation was found to affect the amount of this activity that entered the gradient with tonoplast. These results do not rule out a possible association of acid phosphatase with tonoplast in vivo, but, at the same time, they do suggest the possibility of artifactual association during isolation. Numerous reports in the literature support the existence of tonoplast bound phosphatase as detected by cytochemical staining (18). Examination of purified *Hippeastrum* tonoplast for α-mannosidase activity, a yeast tonoplast marker (24), gave negative results. The same conclusion has been reached for tobacco culture cell tonoplast (4). RNase and DNAse, vacuolar sap constituents of *Hippeastrum* (6), and *Tulipa* (unpublished) are also absent from isolated tonoplast (data not shown). Earlier studies showed that vacuole preparations similar to those used here to purify tonoplast lacked Chi, NADH Cyt c reductase and Cyt c oxidase (25). Starch which accompanied vacuoles was pelleted during density centrifugation.

Recently it was reported (1) that vacuoles isolated from *Vicia faba* protoplasts by osmotic shock in phosphate buffer retained a rind of plasma membrane and cytosol. While the few vacuoles shown by Admon and Jacoby (1) were seriously contaminated, the method used by these workers to distinguish naked from impure vacuoles was tested with *Hippeastrum* and *Tulipa* vacuoles. Perhaps 5% of vacuoles isolated as described (25) showed some evidence of staining with fluorescein-diacetate. In no case did the staining of vacuoles clearly describe the presence of the rind suggested by Admon and Jacoby (1) or clearly observed with intact protoplasts.

The recovery of ATPase realized upon purification of tonoplast by equilibrium density centrifugation on sucrose was 10 to 20%. This loss, which is not understood, occurred when gradients were centrifuged at 4°C or 20°C and has also been observed with *Tulipa* petal and wheat leaf tonoplast. It was previously shown that 70% of *Hippeastrum* vacuole ATPase was recovered after brief centrifugation through a 15% sucrose zone to a 15 to 35% sucrose interface (17). It is also noteworthy that fixation of vacuoles with fluorescein mercuric acetate (26) in 0.7 M mannitol results in hardened membrane ghosts. When fragmented, the membrane equilibrates to a density of 1.17 g per cubic centimeter in a sucrose gradient.

*Hippeastrum* tonoplast was examined directly for the possible presence of UDP-glucose, anthocyanidin-3-O-glucosyl transferase activity. Anthocyanin is known to be restricted to the vacuole in mature plant cells (25), and, therefore, its synthesis might efficiently take place on or in the tonoplast. This location would be particularly expected for the glucosyl transferase activity which is at the end of the anthocyanin biosynthetic sequence. However, earlier studies using whole vacuoles indicated that this organelle lacks three enzymes, including the transferase, which are involved in anthocyanin biosynthesis. All three activities were largely restricted to the enriched cytosol fraction of *Hippeastrum* (12). These observations were extended in this study by examining tonoplast directly for glucosyl transferase activity. No activity was found with tonoplast while, as before, this enzyme was easily demonstrated in enriched cytosol. To test whether this enzyme may have been leached from tonoplast during isolation in 0.2 M K₂HPO₄ (ionic strength, 1.2), a comparison was made between tonoplast prepared by this method and that from vacuoles prepared by gentle osmotic shock induced by lowering the mannitol concentration of a vacuole suspension. The mannitol concentration was lowered from 0.7 M to 0.35 M, and then the suspension was gently passed through a pipette several times. The vacuoles obtained by the latter procedure were visibly contaminated but were reasonably enriched for purified membrane preparation. Both preparations lacked transferase activity. Thus, there continues to be no evidence for the association with vacuoles of enzymes close to anthocyanin biosynthesis. Numerous examples are known of proteins which are loosely associated with membranes, and further study may reveal weak association with the tonoplast of enzymes involved in flavonoid biosynthesis. Weak membrane association can play a role in enzyme regulation, as shown for erythrocyte phosphofructokinase and aldolase (14, 20). These arguments may also apply for vacuole acid phosphatase discussed earlier and other vacuole and cytosol proteins. Cinnamate-4-hydroxylase, an enzyme involved in the formation of p-coumarate (a substrate for flavanone synthase), has been shown to be ER bound (see Ref. 19).

The sensitivity of plant acid phosphatase to fluoride and its insensitivity to EDTA, Ca, and Mg have been described by several workers (Banthorpe *et al.* and references thereof (3). Conversely, ATPases require divalent cations for activity. The results obtained with *Hippeastrum* vacuolar PNPase are consistent with observations of others that KF inhibits while EDTA is not effective or slightly stimulatory. Vacuolar ATPase was inactivated by 2 mM EDTA. In contrast with results obtained with extracts of *T. vulgare* (3), Mg-ATPase was less inhibited by KF than was PNPase. The level of PNPase inhibition by KF was comparable to that.
observed by Torriani (23) for E. coli enzyme. Analysis of the protein character of Hippeastrum tonoplast by SDS-PAGE revealed the presence of five major polypeptides and several minor ones. No evidence was found to support the presence of glycoproteins. Correlation was found between the molecular weights of several minor tonoplast peptides and those of the α and β subunits of CF. In preliminary experiments, treatment of membrane with 0.075% Na deoxycholate released polypeptides having mol wt of 65 and 57 kdaltons, similar to those of the major subunits of coupling factor. Membrane peptides having mol wt of about 46 and 55 kdaltons were also observed. Actin (42 to 43 kdaltons) and a 55-kdalton protein are characteristic of actin containing preparations (8). F actin has been identified as a component of transvacuolar strands of Amaryllis belladonna, a close relative of Hippeastrum (7). Further work is required to determine whether the polypeptides observed are related to coupling factor and contractile proteins.

It was hoped that the sensitivity of detecting membrane proteins on gels could be reproducibly enhanced by lactoperoxidase catalyzed iodination of membrane proteins. However, iodination, while increasing the sensitivity of detection by a factor of 5 to 10, gave inconsistent results, making the method less attractive than detection by Coomassie blue staining. In addition, topographic labeling with lactoperoxidase was precluded by the finding of peroxidase in the vacuole sap (data not shown).

The extensibility of the tonoplast is shown in Figure 7. Tulipa petal vacuoles were allowed to settle on a depression slide coated with 50% carbowax 1540 (Union Carbide Co., New York, NY), 0.1 M glucose, 10 mM CaCl₂, 0.7 mM K-phosphate (pH 8), causing them to adhere to the bottom of the well and occasionally fuse (Fig. 7). After applying a coverslip, additional carbowax solution was infiltrated into the well. The moving liquid caused attached vacuoles to stretch to great lengths. As seen in Figure 7, pigment was retained. Cessation of movement resulted in relaxation of vacuoles to their original size and shape. This extensibility, in contrast to the lack of it in protoplasts (plasma membrane), reflects a unique property of this membrane and undoubtedly is an important characteristic underlying the ability of vacuoles to form transvacuolar strands.

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LITERATURE CITED