Plant Physiol. (1989) 91, 34–38
0032-0889/89/91/0034/05/$01.00/0

Received for publication May 10, 1989
and in revised form June 16, 1989

Communication

Purification of an H^+-Translocating Inorganic Pyrophosphatase from Vacuole Membranes of Red Beet[1]

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ABSTRACT

An H^+-translocating inorganic pyrophosphatase (PPase) was isolated and purified from red beet (*Beta vulgaris* L.) tonoplast. One major polypeptide of molecular weight 67 kilodalton copurified with fluoride-inhibitable PPase activity when subjected to one-dimensional polyacrylamide gel electrophoresis. Overall, a 150-fold purification of the PPase was obtained, from the tonoplast fraction, through anion exchange chromatography of the detergent-solubilized membranes followed by ammonium sulfate precipitation and gel filtration chromatography. The purified polypeptide showed no cross-reactivity with antibodies raised against the 67 kilodalton subunit of the tonoplast ATPase.

The existence of two distinct H^+-translocating pumps, an ATPase and a PPase,[2] both potentially contributing to the electrochemical gradient across the vacuolar membrane, has now been well documented. The enzymes have been shown to be separable by chromatography after detergent-solubilization of tonoplast vesicles isolated from red beet (15) and from the CAM plant *Kalanchoë daigremontia* (3). Both proton pumps caused accumulation of neutral red in individual vacuoles of *Nitella* (17), and in a patch-clamp study of sugar beet vacuoles, Hedrich and Kurkdjian (4) demonstrated the presence of the two electrogenic pumps by measuring membrane potentials induced by ATP and/or PPi in the same vacuole.

As a first step toward a more complete molecular analysis of the protein, it was necessary to purify the PPase and elucidate its polypeptide composition. A recent study which reported the purification of the PPase from microsomal fractions of corn seedlings (12) could not, however, be applied to our system of purified tonoplast from red beet, and we have therefore developed an alternative purification scheme.

[1] Supported by the Natural Sciences and Engineering Research Council of Canada and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche of Quebec.

[2] Abbreviations: PPase, pyrophosphatase; BTP, bis-tris propane; Chaps, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; EB, elution buffer; RB, running buffer; SM, suspension medium.

MATERIALS AND METHODS

Tonoplast Preparation

Fresh red beets (*Beta vulgaris* L.) were bought commercially, kept at 4°C and used within 24 h.

Tonoplast vesicles were prepared as described (14) with some modifications. Peeled and diced beets (350 g) were homogenized at 4°C in a blender with two 30-s bursts in 350 mL homogenization buffer containing 10 mM glycerophosphate, 0.65 M ethanolamine (adjusted to pH 8.0 with concentrated H_2SO_4), 0.28 M choline chloride, 25 mM K-metabisulfite, 3 mM BTP-EDTA, 0.2% BSA (fraction V, essentially fatty acid-free), 10% (w/v) insoluble PVP, 5 mM DTT, and 1 mM PMSF in 70 mM BTP-Mes (pH 8.0). The homogenate was filtered through cheesecloth and centrifuged at 80,000*g* for 30 min in a Beckman Type 45 Ti rotor. The pellets were resuspended with a Dounce homogenizer in a small volume of SM containing 10% (w/v) glycerol, 5 mM BTP-Mes (pH 8.0), 1 mM BTP-EDTA, and 5 mM DTT, then made up to 50 mL, mixed with an equal volume of 0.8 M KI, and centrifuged as above. The KI-treated pellets were resuspended in SM and homogenized, and 7.5 mL aliquots were layered on three step gradients of 10 mL 10% sucrose (w/w) and 20 mL 23% sucrose (w/w) in SM. After centrifugation at 80,000*g* for 2 h in a Beckman SW 28 rotor, the 10/23% interfaces were collected, diluted sevenfold with SM, and sedimented at 80,000*g* for 30 min. The pellets were resuspended in 2 to 3 mL of the same solution and stored at −70°C.

The identification of the 10/23% sucrose gradient interface as the tonoplast fraction has already been documented (14).

Solubilization

Tonoplast aliquots (6–8 mg protein) were pelleted at 80,000*g* for 30 min in a Beckman SW 60 rotor and the pellets resuspended in 2 mL of a solution containing 2.5% Triton X-100, 10% (w/v) glycerol, 30 mM BTP-Mes (pH 8.0), 4 mM $MgCl_2$, 1 mM BTP-EDTA, and 5 mM DTT. After five or six strokes in a Dounce homogenizer, the mixture was incubated on ice for 1 h on an orbital shaker at 100 rpm and homogenized again. The supernatant from a 200,000*g* spin for 40 min in the same rotor was assumed to contain the solubilized enzyme.

PPase and ATPase activities

PPase and ATPase activities were measured by the method of Ames (1), as described (14). Phospholipids were prepared

from partially purified soybean phospholipids (Type IV from Sigma) by sonication (11).

Anion-Exchange Chromatography

A 1.5 cm × 10 cm column was filled with 20 mL DEAE-Sephacel (Pharmacia Canada Ltd.), washed with 50 mL of 1 M KCl, followed by 50 mL of 0.25 M His/Cl (pH 6.0), and finally equilibrated with an elution buffer (EB) containing 20 mM His/Cl (pH 6.0), 10% (w/v) glycerol, 0.25% Triton X-100, 40 mM KCl, 2 mM $MgCl_2$, and 5 mM DTT.

Two mL of solubilized tonoplast was diluted 10-fold with EB without Triton X-100 (to equalize detergent concentrations) but with 0.5 mg/mL phospholipid, and the sample was loaded on the column at a rate of 20 mL/h, followed by a wash with 10 mL EB + 0.5 mg/mL phospholipid. Proteins were eluted with 50 mL of a linear KCl gradient (40–300 mM in EB + 0.5 mM phospholipid) and collected in 1.6 mL fractions in test tubes preloaded with 100 μL EB + 8 mg/mL phospholipid. The column was regenerated with 1 M KCl.

Ammonium Sulfate Precipitation

Fractions from the anion-exchange column containing the highest PPase activities were pooled, sufficient $(NH_4)_2SO_4$ was added to bring the solution to 30% saturation, and the solution was mixed. After incubation on ice for 10 min (shaking), the mixture (6–8 mL) was centrifuged at 10,000*g* for 10 min in a Sorvall RC-5. The supernatant was aspirated off and the pellet resuspended in 0.8 mL of gel filtration running buffer.

Gel Filtration Chromatography

A 1 cm × 100 cm column was packed with Sephacryl S400HR (Pharmacia Canada Ltd.) at 60 mL/h and was equilibrated with RB containing 1% Chaps, 30 mM BTP-Mes (pH 8.0), 10% (w/v) glycerol, 25 mM KCl, 4 mM $MgCl_2$, 1 mM BTP-EDTA, 10 mM DTT, 0.03% (w/v) NaN_3, and 2.0 mg/mL phospholipid. Between 0.1 and 0.2 mg protein from the $(NH_4)_2SO_4$ purified fraction was applied to the column and eluted with RB at 5 mL/h at 2°C.

Preparation of Samples for SDS-PAGE

Fractions (1.25 mL) eluted from the gel filtration column were assayed for PPase activity, pooled in groups of three, and precipitated in 15 mL Corex tubes with ice cold TCA at a final concentration of 15%. After a 30 min incubation on ice, the samples were spun at 10,000*g* for 15 min and the supernatants aspirated off. Twenty μL of sample buffer (10% glycerol, 10% SDS, 100 mM DTT, 63 mM Tris-Cl [pH 6.8], and bromphenol blue) diluted fivefold were added to each tube and the pellets resuspended by thorough vortexing and swirling. Since enough acid usually remained at the bottom of the tubes to turn the bromphenol blue to yellow, small aliquots (2–3 μL) of 1 M Tris were added to the tubes and swirled until the tubes turned blue. The samples were transferred to 1.5 mL microfuge tubes and stored overnight at 4°C; the volumes were then reduced to approximately 20 μL by vacuum centrifugation (Speed-Vac, Savant Instruments) and electrophoresed directly.

SDS-PAGE and Western Blot

One-dimensional SDS-PAGE was performed as described by Laemmli (7). Ten percent acrylamide gels were run in a Mini-Protean apparatus (Bio-Rad). Protein was detected by Coomassie blue staining. Mol wt standards were purchased as a kit (High MW Standards, Sigma). Western blotting was carried out in a Mini Trans-Blot apparatus (Bio-Rad).

Densitometry

An LKB Ultrascan XL Laser Densitometer was used to obtain quantitative density profiles of the Coomassie stained acrylamide gels.

Protein

Protein was measured by the method of Lowry *et al.* (10), after treatment of the samples by the method of Wessel and Flügge (20).

Reagents

Sephacryl S400HR and DEAE-Sephacel were purchased from Pharmacia (Canada) Ltd., Chaps from Pierce Chemical Co., DTT and histidine from ICN Canada Ltd., and Mes from Boehringer Mannheim Canada Ltée. All other reagents were purchased from either Sigma Chemical Co. or BDH Chemicals, Canada.

RESULTS

Anion-Exchange Chromatography

Six to 8 mg of solubilized membranes were loaded onto the DEAE-Sephacel column and eluted with a linear KCl gradient (40–300 mM) as described in "Materials and Methods." Figure 1 shows that the PPase eluted in a sharp peak between 80 and

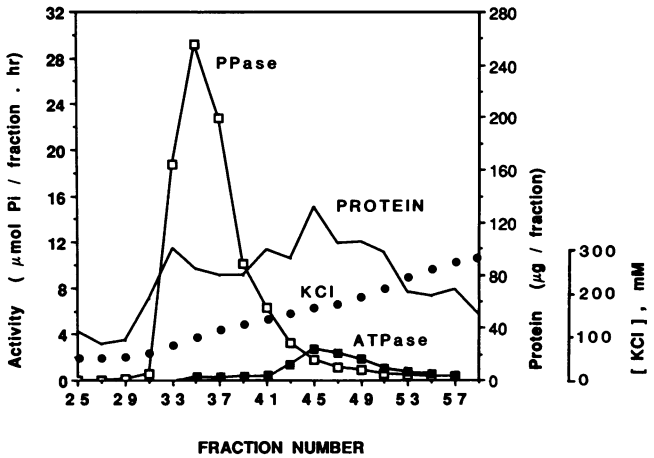

Figure 1. Elution profile from DEAE-Sephacel chromatography of Triton X-100 solubilized tonoplast membranes. PPase (□) and ATPase (■) activities were assayed in a medium containing 100 mM KCl, 30 mM BTP-Mes (pH 8.0), 0.6 mM K-PPi, and 0.5 mM $MgCl_2$ at 37°C. KCl concentrations (●) were measured by chloridometry. Fraction number 25 corresponds to the beginning of the KCl gradient application.

120 mM and was separated from the ATPase activity which eluted between 200 and 250 mM KCl. Phospholipids had to be present at all stages in order to prevent denaturation of the PPase. Since phospholipids appeared to bind the column and elute later than the PPase, it was essential to have additional phospholipids in the tubes where the fractions were collected.

The anion-exchange chromatography was performed at pH 6.0. Initial attempts to run the column at pH 8.0, the optimal pH for activity of the enzyme, resulted in almost complete irreversible inactivation (1.4% activity recovered). At pH 7.0, 27% activity was recovered and at pH 6.0, 100% of the PPi hydrolytic activity applied was accounted for. The enzyme was always assayed at pH 8.0.

Ammonium Sulfate Precipitation

After anion-exchange chromatography, $(NH_4)_2SO_4$ precipitation was used to further purify and concentrate the enzyme. With $(NH_4)_2SO_4$ at a saturation level of 30%, about one-third of the protein and 50% of the PPase activity were precipitated (Table I).

Inhibitor Sensitivity

Sensitivity of the PPi hydrolysis to the PPase specific inhibitor F^- (5, 16) was measured on the solubilized fraction and after the final purification step of the PPase. Residual activity after treatment with 10 mM KF of the solubilized and purified enzymes were 26.3 and 15.4%, respectively, confirming that the purified phosphohydrolytic activity was that of the PPase.

Gel Filtration on Sephacryl S400HR

A further 10-fold increase in specific activity was obtained in the peak PPase fractions after gel filtration (Table I). This additional purification was made possible by the replacement of Triton X-100 with Chaps in the RB which resulted in the relatively earlier elution of the PPase giving a better separation from other proteins. It was also found that having Chaps in the samples applied to the SDS-PAGE produced less distortion of the bands than Triton X-100.

Balance Sheet

The various purification steps resulted in an approximately 150-fold purification of the PPase from the KI-treated tonoplast membranes. The specific activity increased from 13.3 μmol Pi·mg^{-1} protein·h^{-1} to near 2000 μmol Pi·mg^{-1} protein·h^{-1} (Table I). The protein estimate in the peak fraction was made by comparing the staining density on the SDS gel with the mol wt standards whose concentrations were known.

SDS-PAGE

Electrophoresis of the fractions collected from the gel filtration step revealed a polypeptide of mol wt 67 kD which copurified with the PPase activity (Fig. 2). A correlation coefficient of $r = 0.97$ was obtained between the enzyme activities in each lane and the density of the 67 kD band, determined by densitometry. Other bands, such as those around 115 kD or at 60 kD, showed weak correlations with activity; they did not, however, consistently copurify with PPase activity.

Western Blot

The association of PPase activity with a 67 kD polypeptide raised the possibility that this activity could be due to the subunit of the ATPase of the same size (11) which after passage through the anion-exchange column could conceivably be modified to hydrolyze PPi. To examine that possibility, an immunodetection assay was performed against a Western blot from an SDS-PAGE of the fractions eluted from the anion-exchange column. Antibodies raised previously against the 67 kD subunit of the ATPase showed no cross-reactivity with polypeptides from the fractions where the PPase was found (data not shown).

DISCUSSION

Purification of the H^+-translocating inorganic PPase from red beet vacuolar membranes indicates that the catalytic activity of this enzyme is associated with one polypeptide

Table I. *Balance Sheet of PPase Purification from Red Beet Tonoplast*

Tonoplast vesicles were solubilized in 2.5% Triton X-100 and subjected to DEAE-Sephacel chromatography. Peak PPase containing fractions were combined and precipitated with $(NH_4)_2SO_4$ up to a saturation level of 30%. The pellet was resuspended in 0.8 mL of gel filtration running buffer and applied to a Sephacryl S400HR column. Eluting fractions were assayed for PPase activity, grouped in threes, and subjected to SDS-PAGE. Lane 4 refers to Figure 2. Protein in lane 4 was estimated by comparing staining densities of the bands with those of the mol wt standards whose concentrations were known.

Fraction	Protein		PPase activity		
	mg	%	*μmol/h*	%	*μmol/mg·h*
Tonoplast	6.98	100.0	93.0	100.0	13.3
Triton X-100 solubilized	4.62	65.7	160.3	172.3	34.7
DEAE-Sephacel: total	3.05	43.6	157.6	169.5	51.7
DEAE peak fractions	0.66	9.4	85.9	92.4	130.2
Ammonium sulfate	0.23	3.3	43.1	46.3	187.4
Sephacryl S-400: total	N/D[a]		21.0	22.6	
Peak (lane 4 of Fig. 2)	≈0.004	≈0.06	8.1	8.7	≈2000

[a] Not determined.

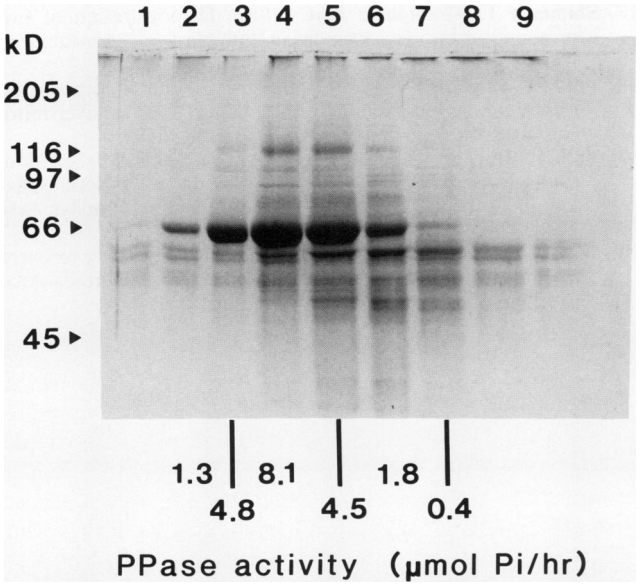

Figure 2. Identification of the 67 kD polypeptide by correlation with PPase activity. Fractions (1 mL) collected from Sephacryl S400HR chromatography were assayed for PPase activity, combined in groups of three and subjected to SDS-PAGE (lanes 1–9). Numbers at the bottom represent total PPase activities in the fractions applied to the corresponding lanes. Mol wt standards are shown on the left. Staining densities were quantified by densitometry and correlated with enzyme activities.

species of 67 kD. This activity is inhibited by F^-, a PPase inhibitor. The estimated mol wt of the PPase is close to that of the 64 kD polypeptide isolated from corn seedling microsomal membranes (12). The origin of their enzyme, however, appears uncertain since they collected their membranes from a 33/46% sucrose interface, where most cell membranes are found. Among other H^+-coupled PPases so far characterized, the photosynthetic bacterium *Rhodospirillum rubrum* has been tentatively assigned two subunits of 54 and 52 kD (2); from rat liver mitochondrial PPase II, two subunits of 28 and 34 kD have been identified (18); and from bovine heart mitochondria subunits of 28, 30, 40, and 60 kD have been reported (19). In the latter enzyme, the authors suggest that the catalytic activity resides in the smaller subunits and therefore no significant similarities would be expected, based on subunit size, between red beet tonoplast PPase and other membrane-bound PPases. As for soluble PPases, their subunit sizes range from 42 kD in sorghum chloroplasts (6) to 19.5 kD in *E. coli* (8), and any similarities with the membrane type PPases will have to await more detailed structural analyses of these polypeptides. Although we cannot yet predict with accuracy the size of the holoenzyme, an approximate size of the protein-detergent-lipid complex of 250 ± 50 kD has been estimated from the gel filtration experiments.

Membrane preparations were treated with KI to reduce contamination by acid phosphatases (13). As well, Lai *et al.* (9) showed that in oat roots, 75% of ATPase activity and approximately 35% of total membrane protein was removed by 0.4 M KI. We did not observe any concomitant loss of PPase activity in red beet. This step was therefore routinely used to increase the specific activity of the starting material.

This final, rather straightforward purification scheme took some time to develop because of the instability of the enzyme once isolated from the native membrane. As shown before (15), the solubilized PPase required the presence of detergent, glycerol, and phospholipids at all stages of its purification in order to retain its activity.

Initially, when preparing samples for SDS-PAGE, phospholipids and detergent were removed from the TCA-precipitated pellets by washing with acetone followed by ethanol or by using the method of Wessel and Flügge (20), which involves partitioning the components in a methanol/chloroform/water mixture. After either treatment, the samples were placed in a 95°C bath for 5 min before applying them to the gel. These procedures were, however, abandoned when it became evident that the ATPase was precipitating and aggregating irreversibly in organic solvents or when heated.

ACKNOWLEDGMENTS

We thank Drs. Morris Manolson and Steven Randall for their valuable advice during the course of this study as well as Karen Ketchum and Heike Pöpperl for critically reviewing the manuscript.

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