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Identification and purification of substrate-binding subunit of higher plant H^+-translocating inorganic pyrophosphatase

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The H^+-translocating inorganic pyrophosphatase (H^+-PPase) of *Beta* vacuolar membrane (tonoplast) vesicles has been purified by 90-fold from detergent-solubilized membranes and the $MgPP_i$-binding subunit identified by affinity labeling. The purified enzyme has a specific activity of 1100 μmol/mg·h and contains one prevalent M_r = 64000 polypeptide which strictly copurifies with activity. Treatment of tonoplast vesicles with *N*-ethylmaleimide (NEM) inhibits the PPase with pseudo-first-order kinetics. Inclusion of $MgPP_i$ in the reaction medium diminishes the pseudo-first-order rate constant (k^0) by more than 30-fold while free PP_i increases k^0 by about 2-fold. Pretreatment of tonoplast vesicles with [^{12}C]NEM in the presence of $MgPP_i$ followed by incubation with [^{14}C]NEM in the presence of $MgPP_i$ or free PP_i yields a single polypeptide of M_r = 64000 which shows $MgPP_i$-protectable, PP_i-potentiated ^{14}C-labeling and comigration with PPase activity during gel filtration. It is deduced that the M_r = 64000 polypeptide constitutes the $MgPP_i$-binding subunit of the H^+-PPase.

Purification; Substrate-binding subunit; Tonoplast; Vacuolar membrane; Pyrophosphatase, H^+-translocating inorganic

1. INTRODUCTION

Tonoplast (vacuolar membrane) energization in higher plants is catalysed by two electrogenic H^+ pumps: an anion-sensitive H^+-ATPase (EC 3.6.1.3) and a cation-sensitive inorganic pyrophosphatase (H^+-PPase; EC 3.6.1.1) [1]. The two pumps are ubiquitous in the vacuolar membranes of higher plants and both are capable of establishing and maintaining the requisite transtonoplast H^+-electrochemical potential difference for H^+-coupled solute transport at the tonoplast.

The tonoplast H^+-ATPase is now well characterized. The major subunits of the enzyme have been identified, the gross partitioning of function between the subunits has been elucidated and some of the genes encoding the component polypeptides have been cloned [1,2]. By comparison, our understanding of the tonoplast PPase is still rudimentary. Potassium ion-stimulated $MgPP_i$ hydrolysis at the tonoplast was initially demonstrated in 1981 [3] and the capacity of the PPase of tonoplast vesicles for H^+-translocation was first shown in 1985 [4]. However, although partial purification and chromatographic resolution of the PPase from the tonoplast ATPase has been achieved [5,6], the polypeptide composition of the PPase is not known. Malsowski and Maslowska [7] claim to have isolated the H^+-PPase from *Triticum* but do not provide an unequivocal demonstration of subunit composition (see section 4). Investigations of the protein chemistry and molecular biology of the H^+-PPase have consequently been prohibited by the lack of suitable material for the preparation of immunological and molecular biological probes. Thus, in this communication we describe the identification of the $MgPP_i$-binding subunit of the H^+-PPase of red beet (*Beta vulgaris* L.) tonoplast vesicles by two independent criteria: substrate-protectable affinity labeling and protein purifica-

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tion. Preliminary reports of our findings have appeared elsewhere [8,9].

2. MATERIALS AND METHODS

2.1. *Materials*

Tonoplast vesicles were isolated from red beet storage root by differential and sucrose density gradient centrifugation [4]. Sephacryl S400 and Mono-Q were purchased from Pharmacia Biotechnology Inc. (Milton Keynes, England) and *N*-ethyl [2,3-^{14}C]maleimide (5 mCi/mmol) from Amersham International, PLC (Amersham, England). All of the general laboratory reagents were from Sigma Chemical Co. Ltd (Poole, England), BDH (Poole, England) or FSA Laboratory Supplies (Loughborough, England). The phospholipids used in these investigations were mixed soybean phospholipids (L-α-phosphatidylcholine, Type IV-S) from Sigma.

2.2. *Membrane solubilization and chromatography*

Tonoplast vesicles were solubilized with Triton X-100 as described previously [5] and the solubilized preparation was incubated on ice for 30 min before chromatography.

The PPase was purified by gel filtration on Sephacryl S400 and anion-exchange FPLC on Mono-Q. A 100 × 1 cm inner diameter column packed with Sephacryl S400 was equilibrated with running buffer (10% (v/v) glycerol, 0.3 (w/v) Triton X-100, 0.05 mg/ml phospholipid, 5 mM dithiothreitol, 1 mM Tris-EDTA, 4 mM $MgCl_2$ and 5 mM Tris-Mes (pH 8.0)). Triton X-100-solubilized tonoplast (3–5 mg protein) was applied and the column was run at a flow rate of 6 ml/h and a temperature of 4°C. Fractions of 1.2 ml were collected and assayed for PPase and ATPase activity and protein.

The peak PPase fractions from chromatography on Sephacryl S400 were then subjected to FPLC on an HR 5/5 Mono-Q column equilibrated with running buffer (20% (v/v) glycerol, 0.1% (w/v) Triton X-100, 0.05 mg/ml phospholipid, 2 mM dithiothreitol, 1 mM Tris-EDTA, 4 mM $MgCl_2$ and 5 mM Tris-Cl (pH 6.0)). The sample (3–5 ml) was applied with a Superloop (Pharmacia Biotechnology Inc., Milton Keynes, England) and eluted at a flow rate of 0.5 ml/min with a five-phase salt gradient of 0–1 M KCl. One ml fractions were collected and aliquots were assayed for PPase activity and protein.

2.3. *PPase, ATPase and protein estimations*

PPase and ATPase activity were determined by measuring the rate of liberation of P_i from PP_i or ATP, respectively, after the precipitation of Triton X-100 and added phospholipid with ice-cold 5% (w/v) trichloroacetic acid, 2% (w/v) perchloric acid [5]. PPase activity was calculated as half the rate of P_i liberation from PP_i (= μmol PP_i consumed/unit time). Protein was estimated by a modification of the Lowry method [10].

2.4. *Inhibition and labeling by* N-*ethylmaleimide*

The standard mixture for reaction with NEM contained 30 mM Tris-Mes (pH 8.0), [^{12}C]- or [^{14}C]NEM (0–100 μM) and the indicated concentrations of ligands (PP_i and/or Mg^{2+}). Membrane protein was added to the mixture and the mixture was incubated at 0°C for 5 min. For the determination of PPase or ATPase activity, the reaction with NEM was terminated by the addition of 667 μM dithiothreitol and aliquots of the mixture were assayed. Affinity labeling of the tonoplast PPase was conducted by pretreating the membranes with 100 μM [^{12}C]NEM in the presence of 0.3 mM Tris-PP_i and 1.3 mM $MgSO_4$ ('$MgPP_i$'). NEM, dithiothreitol and ligands were removed by two successive 100-fold dilutions of the membranes in suspension medium (1.1 M glycerol, 5 mM Tris-Mes, pH 8.0), centrifugation at 80000 × *g* and resuspension in suspension medium. The membranes were treated with 100 μM [^{14}C]NEM (5 mCi/mmol) in reaction mixture containing 0.3 mM Tris-PP_i ('free PP_i') or 0.3 mM Tris-PP_i + 1.3 mM $MgSO_4$ ('$MgPP_i$') at 0°C for 5 min.

The pseudo-first order rate constants for inhibition by NEM (k^o) were derived by non-linear least squares fitting [11] according to the relationship $\%I = 100(1 - \exp(-k^o t[\mathrm{NEM}]))$, in which $\%I$ is the percentage inhibition of PPase or ATPase activity (% control), t is time and [NEM] is the concentration of NEM.

2.5. *SDS-PAGE and fluorography*

One-dimensional SDS-PAGE was performed as described by Laemmli [12] on concave exponential gradient gels [13]. Detergent and lipid were removed from the fractions before the addition of denaturation buffer by extraction with acetone/ethanol (1 : 1, −20°C). The gels were stained with silver stain (Bio-Rad Laboratories Ltd, Watford, England) or Coomassie blue. ^{14}C-labeled polypeptides were detected fluorographically after destaining the Coomassie blue-stained gels in 7% (v/v) acetic acid. The gels were impregnated with Amplify (Amersham International PLC, Amersham, England), dried and exposed to preflashed X-ray plates at −80°C. Quantitative densitometry was performed by scanning the stained gels with an LKB Bromma Ultroscan XL Enhanced Laser Densitometer at 633 nm.

3. RESULTS

3.1. *Solubilization and purification of PPase*

Chromatography of TX-100-solubilized tonoplast on Sephacryl S400, equilibrated and eluted with running buffer containing 0.3% (w/v) Triton X-100, 0.05 mg/ml phospholipid and 4 mM $MgCl_2$ (fig.1A) results in an approximately 7-fold enrichment of the PPase relative to tonoplast vesicles (table 1). Subsequent FPLC of the peak PPase fractions from the Sephacryl S400 column on Mono-Q (fig.1B) enables a further 12–13-fold enrichment to yield enzyme with a specific activity of 1100 μmol/mg·h (table 1). The overall purification is between 85- and 90-fold with a mean recovery of 40% (table 1).

Previous investigations [5] have established the need for 0.05 mg/ml phospholipid and 4 mM Mg^{2+} in the solubilization medium and column buffers for the quantitative recovery of PPase activity: these components were therefore included

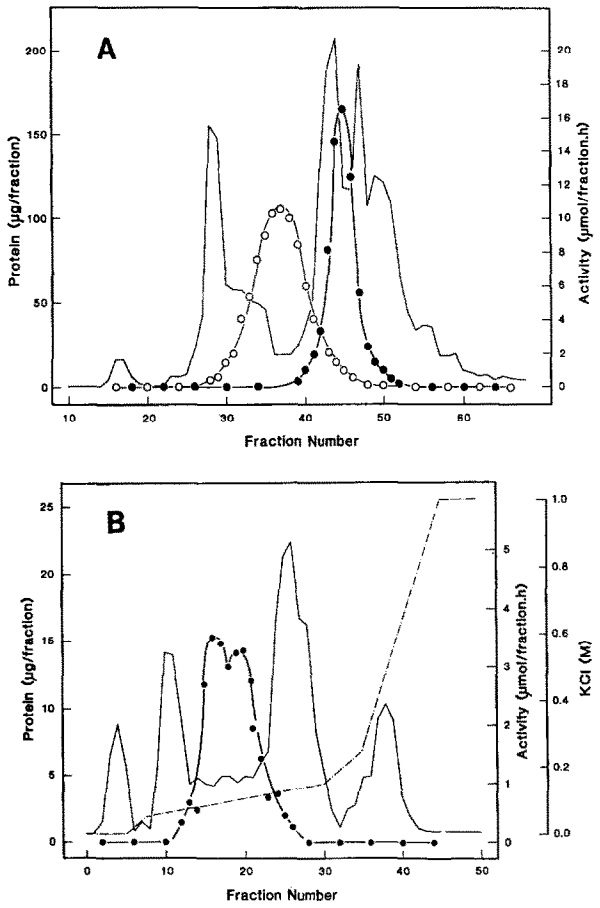

Fig.1. Chromatographic purification of tonoplast PPase. (A) Chromatography of Triton X-100-solubilized tonoplast on Sephacryl S400. Four mg of solubilized protein were applied. (B) FPLC of peak PPase fractions from Sephacryl S400 on Mono-Q. Three ml of sample was applied. PPase activity (●—●), ATPase activity (○—○), protein (——), KCl gradient (- - - -).

throughout the purification to minimize irreversible denaturation of the enzyme. The PPase fractions from chromatography, in addition, require sonicated phospholipid in the assay media for maximal activity. Stimulation by added phospholipid is greatest at 1.3 mg/ml and the peak fractions from both Sephacryl S400 and Mono-Q chromatography are stimulated by 2.2-fold (table 1).

The identity of the purified enzyme with the PPase of tonoplast is confirmed by its ion requirements (table 2). Material from all 3 stages of the purification shows maximal activity with K_2SO_4, KCl and KNO_3. Sodium salts are a poor substitute for K^+ and all three preparations show a strict dependence on Mg^{2+} for activity. The less pronounced Mg^{2+}-activation and lower apparent K^+/Na^+ discrimination of the Mono-Q-purified enzyme is attributed to the carry-over of 0.4 mM Mg^{2+} and approximately 10 mM KCl from the Mono-Q elution buffer.

Table 1

Purification of tonoplast H^+-PPase

Step	Specific activity (µmol/mg·h)		Purification (fold)	Recovery (%) (step)
	+PL	−PL		
Tonoplast	12.9	12.9	1.0	100.0
Sephacryl S400				
Total fractions	–	–	–	96.5
Peak PPase	87.9	40.0	6.8	–
Mono-Q				
Total fractions	–	–	–	42.5
Peak PPase	1100.0	500.0	12.5	–
Overall	–	–	85.3	41.0

The enzyme was purified as described in section 2 and the legend to fig.1. Native membranes were assayed in the presence of 5 µM gramicidin-D to ensure H^+/cation equilibration. The chromatographic fractions were assayed in the presence (+PL) or absence (−PL) of 1.3 mg/ml sonicated phospholipid. 'Peak PPase' refers to the fractions containing the highest PPase activity

Table 2

Ion requirements of tonoplast PPase before and after purification

Salt	Activity (µmol/mg·h)		
	Tonoplast	Sephacryl peak	Mono-Q peak
$MgSO_4$	0.9	6.5	287.4
+ 50 mM KCl	13.9	47.9	885.7
+ 50 mM KNO_3	14.4	51.5	821.4
+ 25 mM K_2SO_4	15.1	65.2	1092.9
+ 50 mM NaCl	3.1	10.9	300.0
− $MgSO_4$	1.3	7.3	442.9

PPase activity was measured in a reaction system containing 0.3 mM Tris-PP_i, 30 mM Tris-Mes (pH 8.0), 5 µM gramicidin-D, 1.3 mg/ml sonicated phospholipid and the indicated concentrations of mineral salts. Unless otherwise indicated, $MgSO_4$ was included at a concentration of 1.3 mM

3.2. *Polypeptide composition*

SDS-PAGE of the PPase-containing fractions from FPLC on Mono-Q (fig.2) reveals a substantial enrichment of a polypeptide of M_r = 64500 ('64000'). Although other bands are discernible at M_r = 86000, 50000, 32000 and 23000, only the M_r = 64000 polypeptide copurifies stoichiometrically with PPase activity. The intense band at M_r = 23000, for instance, is most prominent in those fractions lacking PPase activity. Densitometry of the fractions from FPLC on Mono-Q confirms the correspondence between the intensity of the M_r = 64000 band and PPase activity (fig.3): PPase activity and band intensity are linearly related with a correlation coefficient (Least Squares Method) of 0.95.

3.3. *Inhibition and labeling by* N-*ethylmaleimide*

The PPase activity of native vesicles is subject to irreversible inhibition by NEM (fig.4A). Inhibition is pseudo-first-order: semilog plots of PPase activity (% of control) as a function of NEM concentration are linear. The value of the pseudo-first-order rate constant is, however, markedly affected by the inclusion of $MgPP_i$ or free PP_i in the NEM reaction mixture. Quantitative protection is conferred by $MgPP_i$ (k^o = 0.09 ± 0.08 × 10^3 $M^{-1} \cdot min^{-1}$) whereas free PP_i increases the inhibitory potency of NEM by about 2-fold (k^o = 5.31 ± 0.37 × 10^3 $M^{-1} \cdot min^{-1}$) relative to controls minus ligands (k^o = 2.93 ± 0.10 × 10^3 $M^{-1} \cdot min^{-1}$). Mg^{2+}, alone, decreases k^o by only 20% from 2.93 ± 0.10 × 10^3 to 2.34 ± 0.10 × 10^3 $M^{-1} \cdot min^{-1}$. Neither $MgPP_i$ nor free PP_i affect the kinetics of inhibition of the tonoplast ATPase by NEM: k^o_{ATPase} has an aggregate value of 5.90 ± 0.40 × 10^4 $M^{-1} \cdot min^{-1}$ with no significant difference between treatments (fig.4A, inset).

If the preincubation of tonoplast vesicles with [^{12}C]NEM in the presence of $MgPP_i$ blocks the non-protectable NEM-reactive groups (and preserves the protectable groups) of the enzyme, then subsequent removal of protectant and incubation with [^{14}C]NEM should provide a means of specifically labeling the $MgPP_i$-protectable, NEM-reactive group(s) of the PPase. The results in

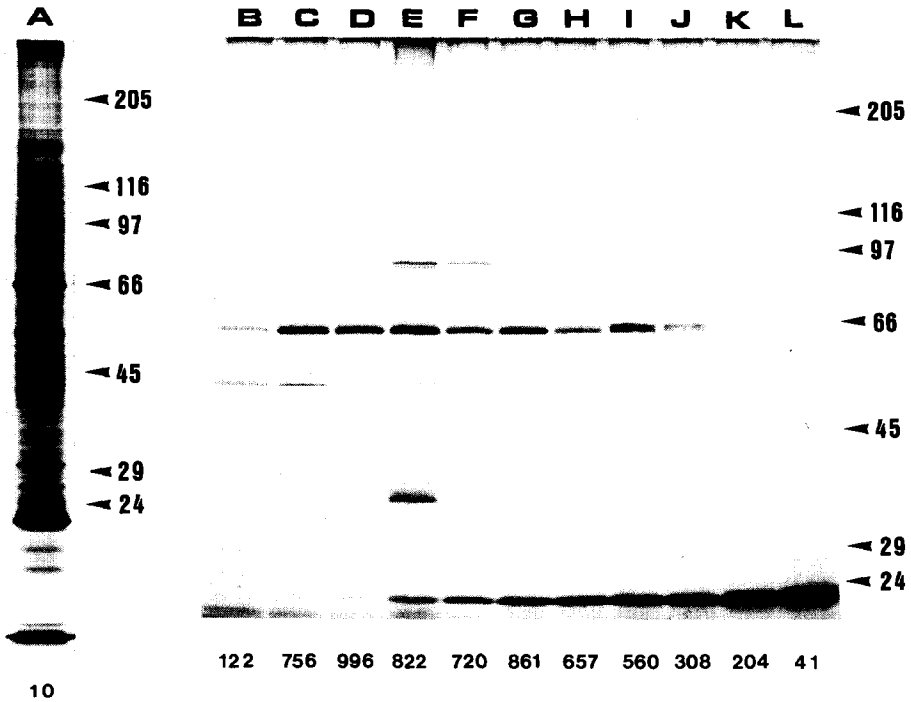

Fig.2. SDS-PAGE of purified PPase. Successive PPase-containing fractions from FPLC on Mono-Q were electrophoresed. The samples (20 μg tonoplast, lane A, and 4 μg aliquots of the Mono-Q fractions, lanes B–K) were run on 7–12% concave exponential gels and the gels were silver-stained. The numbers under the lanes represent the specific activities (μmol//mg·h)) of the corresponding Mono-Q fractions.

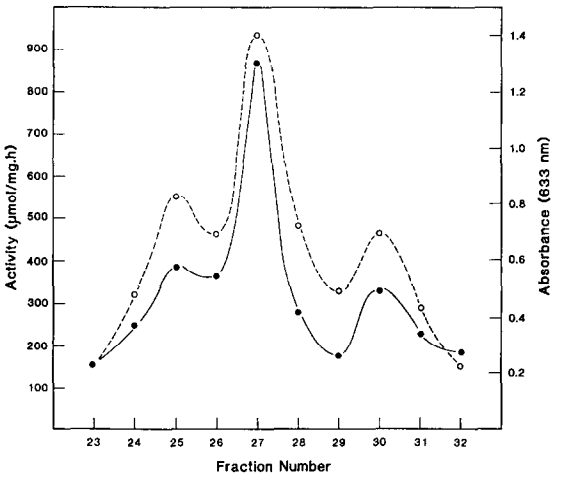

Fig.3. Relationship between intensity of staining of M_r = 64000 band of fractions from FPLC on Mono-Q (●) and PPase activity (○). Aliquots of successive PPase-containing fractions (4 μg/lane) were subjected to SDS-PAGE on 7–12% concave exponential gels and the gels were silver-stained before densitometry at 633 nm. The PPase activities of the fractions were determined before SDS-PAGE as described in section 2.

fig.3B demonstrate the practicability of such an approach. When vesicles are incubated with 100 μM [^{12}C]NEM + $MgPP_i$, washed and allowed to react with 0–100 μM [^{14}C]NEM, the protective action of $MgPP_i$ and potentiating action of PP_i are conserved in the second reaction cycle.

Analysis of tonoplast vesicles labeled with 100 μM [^{14}C]NEM after pretreatment with 100 μM [^{12}C]NEM in the presence of $MgPP_i$ reveals two differentially labeled polypeptides of M_r = 64000 and 23000, respectively (fig.4A). The M_r = 64000 polypeptide, alone, is subject to labeling under conditions which cause maximal inhibition of the PPase: labeling is abolished by $MgPP_i$ and potentiated by free PP_i. The M_r = 23000 polypeptide, on the other hand, undergoes most marked labeling by [^{14}C]NEM when the inhibition of enzymic activity is minimal, i.e. when $MgPP_i$ is included in the NEM reaction mixture. Although both the ^{14}C-labeled M_r = 64000 and 23000 polypeptides copurify with activity when tonoplast vesicles labeled with [^{14}C]NEM in the presence of free PP_i

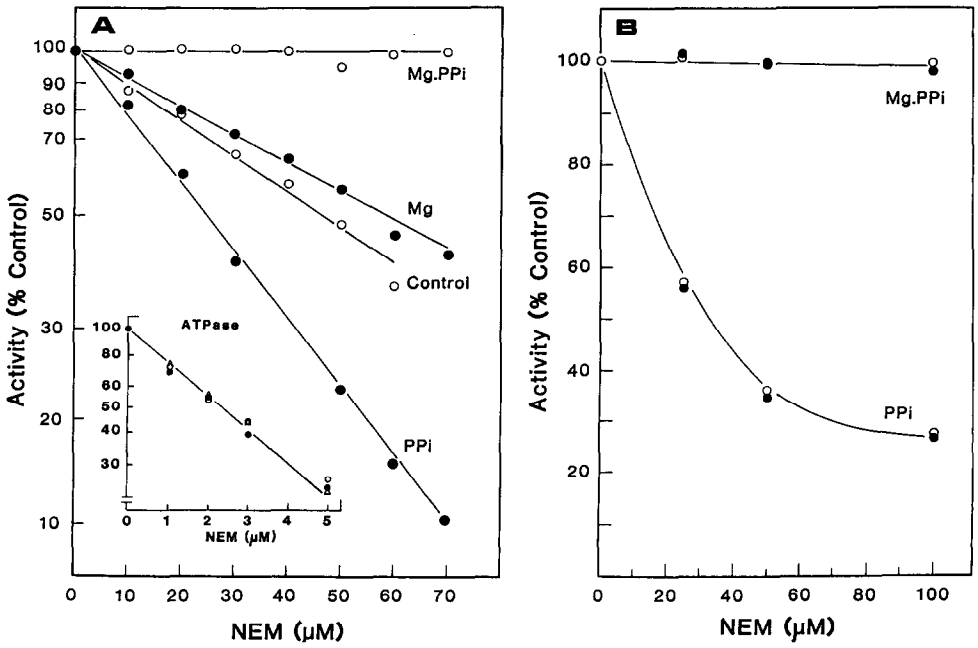

Fig.4. Kinetics of inhibition of tonoplast PPase and ATPase by NEM. (A) Effects of no ligands ('Control'), free Mg^{2+} (1.3 mM $MgSO_4$), free PP_i (0.3 mM Tris-PP_i) and $MgPP_i$ (0.3 mM Tris-PP_i + 1.3 mM $MgSO_4$) on inhibition of tonoplast PPase by NEM. (Inset) Effects of no ligands (○), $MgPP_i$ (●) and free PP_i (△) on inhibition of ATPase by NEM. (B) Effect of pretreatment with [^{12}C]NEM on subsequent inhibition by [^{14}C]NEM. Tonoplast vesicles were pretreated with 0–100 μM [^{12}C]NEM + $MgPP_i$ (●) or free PP_i (●), washed free of ligands, NEM and dithiothreitol and the (100 μM NEM + $MgPP_i$)-pretreated vesicles were incubated with 0–100 μM [^{14}C]NEM in the presence of free PP_i (○) or $MgPP_i$ (○).

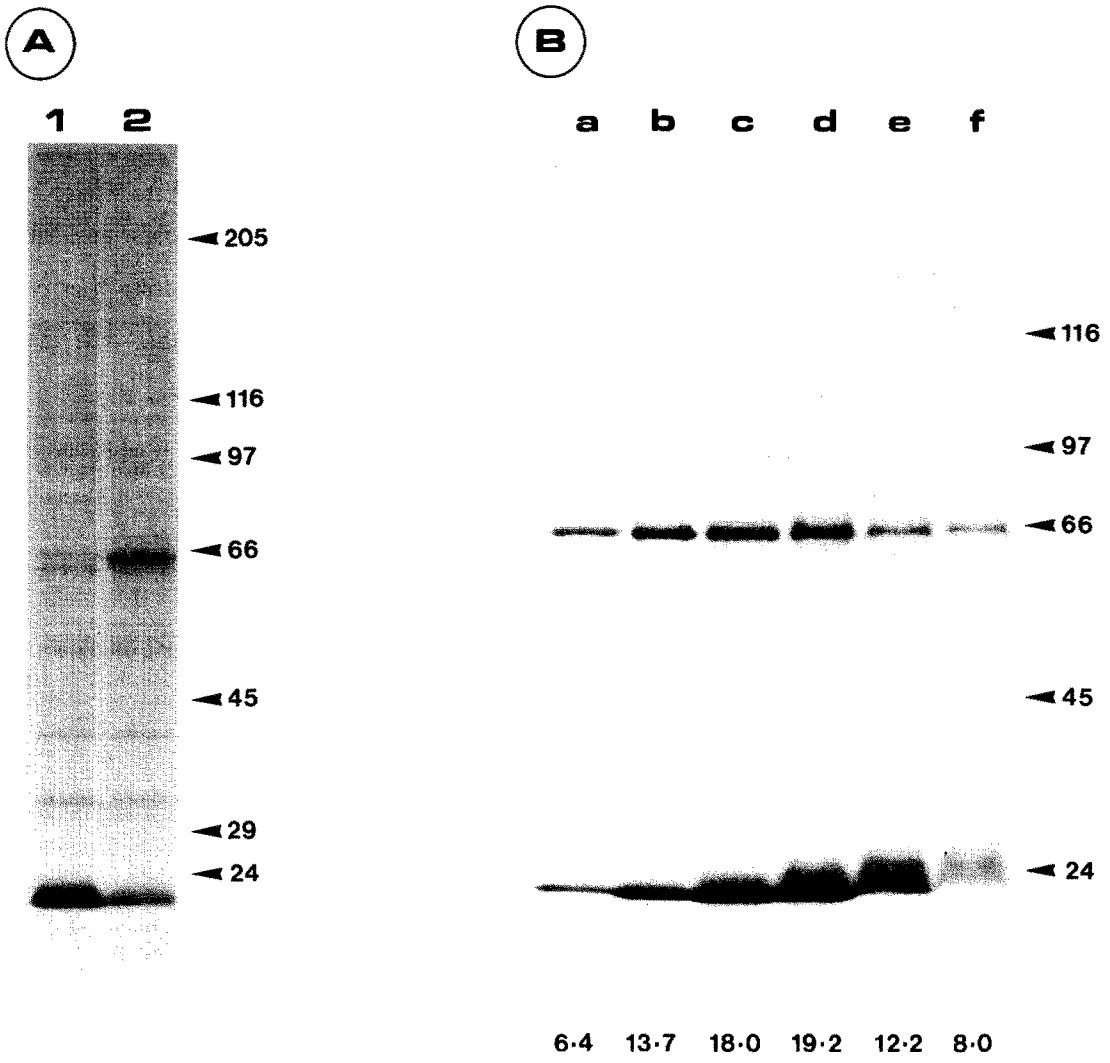

Fig.5. SDS-PAGE and fluorography of [^{14}C]NEM-treated tonoplast and partially purified PPase. (A) ([^{12}C]NEM + $MgPP_i$)-pretreated tonoplast labeled with 100 μM [^{14}C]NEM in the presence of $MgPP_i$ (lane 1) or free PP_i (lane 2). Each lane was loaded with 50 μg protein. (B) Fractions from Sephacryl S400 chromatography of tonoplast labeled with [^{14}C]NEM + free PP_i. ([^{12}C]NEM + $MgPP_i$)-pretreated tonoplast was incubated with 100 μM [^{14}C]NEM + free PP_i, added to an equal amount of untreated tonoplast protein, solubilized with Triton X-100 and subjected to Sephacryl S400 chromatography. Equivalent portions of each of the fractions enclosing the PPase peak were then electrophoresed (lanes a–f). The numbers under the lanes represent the volume activities of PPase (μmol/fraction·h) in the corresponding chromatographic fractions.

are solubilized and chromatographed on Sephacryl S400 (fig.4B), the M_r = 23000 polypeptide is clearly resolved from the PPase during FPLC on Mono-Q (fig.2). The M_r = 23000 polypeptide therefore does not have the characteristics of the $MgPP_i$-binding subunit of the PPase.

Partial purification of the tonoplast ATPase from vesicles labeled with [^{14}C]NEM by the same protocol shows no labeling of the constituent polypeptides of this enzyme. Thus, the conditions for labeling are relatively specific to the PPase, in agreement with the kinetic data (fig.4A, inset).

4. CONCLUSIONS

Malsowski and Maslowska [7] ascribe an M_r = 64000 polypeptide to the microsomal H^+-PPase of *Triticum* but their ascription was based on

purification data alone: parameters relating catalytic function to polypeptide composition were not provided. With no knowledge of the abundance of PPase subunits in the membrane, polypeptides copurifying with enzymic activity might have been misidentified as subunits and/or low-abundance polypeptides specifically associated with the catalytic function of the enzyme might have been overlooked. The data presented above therefore provide the first clear indication of the identity of the $MgPP_i$-binding subunit of the H^+-PPase of higher plant vacuolar membrane. The M_r = 64000 polypeptide, alone, strictly copurifies with the PPase and is subject to $MgPP_i$-protectable and PP_i-potentiated covalent modification by [^{14}C]NEM. Since the inhibition of PPase activity by NEM is also abolished by $MgPP_i$ and potentiated by free PP_i, the M_r = 64000 polypeptide is concluded to carry the NEM-reactive, $MgPP_i$-binding site responsible for inhibition. This polypeptide is therefore tentatively identified as the catalytic subunit of the enzyme.

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