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Dimeric structure of H^+-translocating pyrophosphatase from pumpkin vacuolar membranes

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Vacuolar membrane H^+-translocating pyrophosphatase (H^+-PPase) was purified from pumpkin seedlings. Its enzymatic properties including molecular size of constituting polypeptide (75 kDa) were very similar to those of mung bean H^+-PPase [(1989) J. Biol. Chem. 264, 20068–20073]. The native, functional molecular size of the pumpkin H^+-PPase was estimated to be 135–139 kDa from gel permeation HPLC of the purified enzyme in the presence of detergent and from radiation inactivation of the enzyme in vacuolar membranes. It is concluded that native, functional pumpkin H^+-PPase, and also probably H^+-PPases from other plants, is a dimer of 75 kDa subunits.

H^+-translocating pyrophosphatase; Plant vacuole; Radiation inactivation; Pumpkin

1. INTRODUCTION

The membranes of plant vacuoles have two distinct proton pumps; H^+-translocating ATPase and H^+-translocating pyrophosphatase (H^+-PPase is a rather unique enzyme for plant vacuoles [2–9], although a photosynthetic bacterium, *Rhodospirillum rubrum*, has also been reported to have H^+-PPase [10]. H^+-PPase has been purified from vacuolar membranes of mung bean [7] and red beet [8,9] and consists of a single kind of polypeptide whose molecular size has been estimated to be 73 kDa [7], 67 kDa [8], and 64 kDa [9] from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. However, this does not exclude the possibility that native, functional H^+-PPase in the membranes is an oligomer of this polypeptide. In fact, cross-linking experiments indicated a dimeric structure for H^+-PPase [11]. Also, Chanson and Pilet reported that the functional molecular size of H^+-PPase of maize root vacuolar membranes was about 160 kDa, although they reported much smaller molecular sizes of constituting polypeptides than those reported by others [5]. Here, we report purification of H^+-PPase from pumpkin seedlings, estimation of functional molecular size of H^+-PPase from radiation inactivation experiments, and measurement of native molecular size of H^+-PPase using gel permeation HPLC in the presence of detergent.

Abbreviations: Chaps, 3-[(3-cholamidepropyl)-dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; PPase, pyrophosphatase; SDS, sodium dodecyl sulfate

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2. EXPERIMENTAL

2.1. *Preparation of vacuolar membrane vesicles*

Seeds of pumpkin (*Cucurbita moschata*) were soaked in water for about 5 h with several changes and sown in trays with moist vermiculite. Seedlings were germinated in the dark for 7 days at 25°C. After germination, hypocotyl tissues were washed with water, and soaked in high osmotic buffer containing 50 mM Tris-H_2SO_4 (pH 7.5) and 0.5 M sucrose for 10 min. The tissues were put into a vacuum desiccator, and evacuated for 10 min. Vacuolar membrane preparation and purification of PPase were carried out according to the procedures described by Maeshima [5]. The vacuolar membranes were stored as a suspension in 20 mM Tris-H_2SO_4 (pH 7.5) containing 20% glycerol, 1 mM DTT, 1 mM EGTA, and 2 mM $MgSO_4$ (Tris/GDEM buffer) at a final protein concentration of 1.5 mg/ml at −80°C.

2.2. *Purification of PPase*

Purification of PPase was also carried out according to the procedures described by Maeshima [5]. Solid KCl and 5% deoxycholate were added to the vacuolar membrane suspension at a final concentration of 50 mM and 0.2%, respectively. The suspension was centrifuged at 150 000 × *g* for 1 h. The pellet was suspended in Tris/GDEM buffer containing 0.4% lysophosphatidylcholine to bring the volume to half of the original membrane suspension. The suspension was gently stirred for 20 min at 25°C. After stirring, the suspension was centrifuged at 15 000 × *g* for 40 min at 4°C. The supernatant (lysophosphatidylcholine extract) was applied to a QAE-Toyopearl column equilibrated with Tris/GDEM buffer containing 0.1% Triton X-100. The column was washed with Tris/GDEM buffer containing 0.1% Triton X-100 and 50 mM NaCl. After washing, PPase was eluted from the column with Tris/GDEM buffer containing 0.1% Trixon X-100 and 100 mM NaCl. The eluate (QAE-Toyopearl eluate) contained pure PPase and was stored at −80 °C.

2.3. *Assays*

Unless otherwise stated the PPase reaction was started by adding 10 μl of the enzyme into 200 μl of 1 mM sodium pyrophosphate, 50 mM KCl, 1 mM sodium molybdate, 0.02% Triton X-100, 1 mM $MgCl_2$, 20 μg/ml of suspended phospholipid, and 30 mM Tris-H_2SO_4 (pH 7.2). After incubation for 10 min at 30°C, the reaction was terminated by the addition of 300 μl of 2% perchloric acid. The amount of P_i released was determined by Fiske Subbarow method

[12]. One unit of PPase activity was defined as the amount of enzyme which hydrolyzed 1 μmol of pyrophosphate in one min. Glucose 6-phosphate dehydrogenase was assayed at 25°C in 25 mM Tris-HCl (pH 7.8), 2.5 mM glucose 6-phosphate, and 0.125 mM $NADP^+$ [13]. Protein concentration was determined by the method of Lowry [14] after precipitation by 10% trichloroacetic acid with bovine serum albumin as a standard.

2.4. *Analytical methods*

Polyacrylamide gel electrophoresis was carried out according to Laemmli [15] using 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS). Molecular size of purified H^+-PPase was estimated by gel permeation HPLC on a G3000SW XL column (Tosoh Co., Japan). The column was equilibrated with 30 mM Tris-H_2SO_4 (pH 7.2), 10% glycerol, 50 mM KCl, 0.5% 3-[(3-cholamidepropyl)-dimethylammonio]-1-propanesulfonate (Chaps), 1 mM EGTA, and 2 mM $MgCl_2$. The column was eluted at a flow rate of 0.5 ml/ml at room temperature.

2.5. *Radiation inactivation experiment*

Vacuolar membranes were diluted to 1 mg/ml in Tris/GDEM buffer at 4°C. Glucose 6-phosphate dehydrogenase from yeast (Sigma, grade II) was included at 20 μg/ml as an internal standard with a known molecular mass of 102 kDa [16]. Each 100 μl of the sample suspension was dispensed into micro plastic tubes equilibrated with N_2 gas. The tubes were sealed tightly with caps and Parafilm and frozen immediately in liquid N_2. Samples were placed at a fixed distance from the radiation source, and irradiated with a ^{60}Co γ ray apparatus (6000 Ci) at the Research Center for Nuclear Science and Technology (University of Tokyo). During irradiation, samples were maintained at −77°C with dry ice-methanol. The dose rate of the γ ray was 0.12 Mrad/h as measured with a Fricke dosimeter at room temperature. The irradiated samples were removed at appropriate time intervals and stored at −80 °C. After irradiation, all the samples were thawed and the remaining enzyme activities were assayed. The experiments were repeated twice. The results were analyzed by classical target theory [17]. The molecular sizes of the enzymes were calculated from the following formula [18]: $M_r = 6.4 \times 10^5/D_{37}$ (Mrad) × S_t, where S_t is a temperature correction factor of 2.0 (−77°C) [19] and D_{37} is the radiation dose necessary to decrease the enzyme activity to 37% of the initial activity.

3. RESULTS AND DISCUSSION

3.1. *Solubilization and purification of vacuolar membrane PPase*

The vacuolar membrane PPase was purified from pumpkin by the selective solubilization of vacuolar membrane proteins and QAE-Toyopearl step gradient chromatography. About 30% of proteins contained in vacuolar membranes were solubilized and removed by pretreatment with 0.2% deoxycholate. Then the PPase, together with H^+-ATPase and an unknown 24 kDa protein, was solubilized with 0.4% lysophosphatidylcholine at 25°C. Protein band patterns of vacuolar membranes and the solubilized fraction from pumpkin in SDS-polyacrylamide gel electrophoresis (Fig. 1, lane 1,2) were very similar to those of mung bean seedlings [7]. PPase was separated from other proteins by QAE-Toyopearl chromatography with a step NaCl gradient (Table I). The purified PPase was composed of a single kind of polypeptide the molecular size of which was estimated to be 75 kDa from SDS-polyacrylamide gel electrophoresis (Fig. 1, lane 3). Almost the same values were reported for H^+-PPases from mung bean and red beet [7–9].

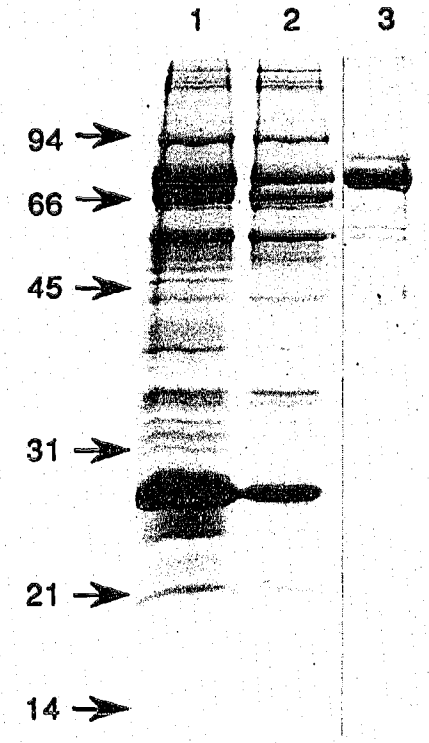

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified PPase from pumpkin. Lane 1, vacuolar membranes (10 μg); lane 2, lysophosphatidylcholine extract (8 μg); lane 3, QAE-Toyopearl eluate (4 μg). The *arrows* indicate the positions of molecular mass standards (in kDa).

3.2. *Enzymatic properties of the pumpkin PPase*

The pumpkin PPase hydrolyzed inorganic pyrophosphate but did not hydrolyze any nucleotide phosphates examined. When the PPase activity was assayed with keeping Mg^{2+}/pyrophosphate in a constant 1:1 ratio, the PPase activity increased until 0.2 mM of Mg-pyrophosphate and then decreased gradually. The half-maximum activity was obtained at about 80 μM Mg-pyrophosphate. Excess Mg^{2+} and pyrophosphate inhib-

Table I

Purification of PPase from pumpkin

Fraction	Total protein (mg)	Total units (U)	Recovery (%)	Specific activity (U/mg)
Vacuolar membranes*	38.4	33.0	100	0.86
Lysophosphatidylcholine extract	20.3	31.5	95.3	1.55
QAE-Toyopearl eluate	0.78	6.32	19.1	8.10

*Purification was started from 1.4 kg of hypocotyl tissues of pumpkin seedlings.

ited PPase activity. PPase activity was dependent on the presence of divalent cations such as Mg^{2+}, Zn^{2+}, Ba^{2+} and Mn^{2+}. However, the enzyme did not utilize Ca^{2+} as co-factor and Ca^{2+} strongly inhibited the activity even in the presence of Mg^{2+}. A monovalent cation was required for the maximum activity. The relative PPase activities at 50 mM K^+, Rb^+, NH_4^+ and Cs^+ were 440, 345, 285 and 135%, respectively (activity without addition of monovalent cations was taken as 100%). Azide (1 mM), nitrate (10 mM) and molybdate (1 mM) had essentially no effect on the enzyme reaction. Vanadate (100 μM) inhibited 40% of the activity. Pretreatment of the enzyme with 100 μM *N*-ethylmaleimide, 100 μM 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole, or 100 μM dicyclohexylcarbodiimide resulted in partial inactivation (50–80%) of the enzyme. Since these properties are commonly observed for plant vacuolar H^+-PPases [3,6,7,20–23], we concluded that the PPase purified from pumpkin vacuolar membranes was a H^+-PPase.

3.3. *Radiation inactivation of PPase*

In order to estimate the functional molecular size in situ, pumpkin vacuolar membranes were irradiated with γ rays. PPase activity decays as a single exponential function of dosage (Fig. 2). The D_{37} value of glucose 6-phosphate dehydrogenase, an internal standard, was 12.4 Mrad ($r = 0.98$) which corresponded to a radiation-sensitive mass of 103 kDa. This value is in good agreement with the actual molecular size of the glucose 6-phosphate dehydrogenase, 102 kDa [16]. The D_{37} value for PPase was 9.23 Mrad ($r = 0.99$) and the functional molecular size of PPase was calculated to be 139 kDa.

3.4. *Gel permeation HPLC*

The native molecular size of the purified pumpkin PPase was estimated with gel permeation HPLC using a G3000 SWXL column equilibrated with a buffer containing 0.5% Chaps. Proteins used as molecular weight standards were also analyzed in the presence of 0.5% Chaps. As shown in Fig. 3, a peak containing PPase

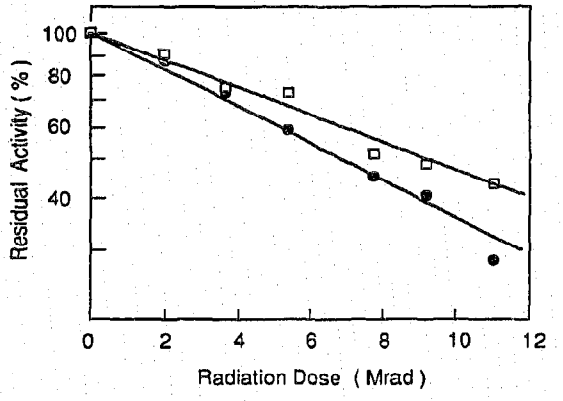

Fig. 2. Radiation inactivation of pumpkin vacuolar PPase. Each point represents an average value of seven assays of three independent experiments. The lines were a least-square fit of the data on a semilog plot. (●), PPase; (□); glucose 6-phosphate dehydrogenase.

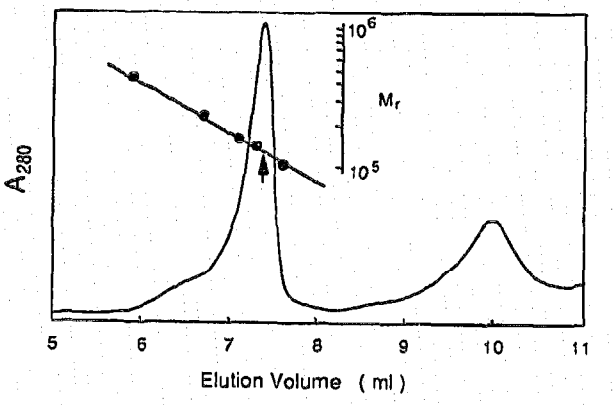

Fig. 3. Gel permeation HPLC of purified PPase in the presence of 0.5% Chaps. Approximately 10 μg of purified pumpkin PPase was into a G3000SWXL column, ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), lactate dehydrogenase from rabbit muscle (140 kDa), and glucose 6-phosphate dehydrogenase from yeast (102 kDa) were used as molecular size standards. The arrow indicates the peak position of PPase. The other conditions are described in section 2.

appeared at elution volume 7.4 ml which was slightly later than lactate dehydrogenase (140 kDa). The molecular size of the peak fraction was estimated to be 135 kDa. There was no peak at the region corresponding to molecular size 75 kDa, a monomer size of PPase. A small peak appeared at elution volume 10 ml. However, judged from SDS polyacrylamide gel electrophoresis, this fraction did not contain protein and was probably Triton X-100 or another component contained in the applied solution.

3.5. *Dimeric structure of the H^+-PPase*

Thus, values of molecular sizes estimated from radiation inactivation and gel permeation HPLC well agreed with each other and we can conclude that native, functional molecular size of pumpkin PPase is 135–139 kDa. Since SDS-polyacrylamide gel electrophoresis of the purified pumpkin PPase showed that the enzyme is made up of 75 kDa polypeptides, the native pumpkin PPase should be a dimer of 75 kDa subunits. Taking into account of common characteristics of vacuolar H^+-PPases from various plants [3,6,7,20–23], our results strongly suggest that plant vacuolar H^+-PPases in general have the same kind of dimeric structure.

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