PERGAMON

Phytochemistry 50 (1999) 535–539

PHYTOCHEMISTRY

Vacuolar H^+-pyrophosphatase purified from pear fruit

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Received 12 March 1998; revised 2 July 1998

Abstract

A vacuolar H^+-translocating inorganic pyrophosphatase was purified from pear fruit through selective detergent treatments, Superose 6 and Mono Q column chromatography. The specific activity of the purified enzyme was 850 μmol h^{-1} mg $protein^{-1}$. The *Mr* of V-PPase was 66 kDa by SDS-PAGE and the polypeptide cross-reacted with the antiserum against V-PPase of mung bean. The purified V-PPase was stimulated by potassium and inhibited by calcium and N, N′-dicyclohexylcarbodiimide.

Keywords: *Pyrus communis*; Rosaceae; Pear fruit; Enzyme purification; Vacuolar H^+-translocating inorganic pyrophosphatase; Tonoplast

1. Introduction

The vacuole is a dynamic organelle involved in several cellular processes including storage of metabolites and ions, regulation of cytosolic homeostasis, degradation and recycling of cellular components, and space filling (Maeshima, Nakanishi, Matsura-Endo & Tanaka, 1996; Taiz, 1992). Many of these processes are directly or indirectly related to either the transmembrane electrochemical gradient across the vacuolar membrane or acidic pH in the vacuole. These gradients are generated by two proton pumps in the vacuolar membrane; the vacuolar H^+-ATPase (V-ATPase) and the vacuolar H^+-pyrophosphatase (V-PPase). V-ATPase from the tonoplast of higher plants has been well characterized (Sze, Ward & Lai, 1992) and purified from the flesh tissue of pear fruit (Hosaka, Kanayama, Shiratake & Yamaki, 1994). V-PPase, which is a universal enzyme among green plants (Maeshima, Miura & Sato, 1994), has also been isolated from various tissues of higher plants (Sarafian & Poole, 1989; Britten, Turner & Rea, 1989; Maeshima & Yoshida, 1989; Sato, Kasahara, Ishii, Homareda, Matsui et al., 1994; Perotti, Gavin, Widmer & Chanson, 1994; Becker, Canut, Luttge, Maeshima, Marigo & Ratajczak, 1995). However, V-PPase has not been isolated from the flesh tissue of fruit where it may play important roles in development.

For approximately forty days after flowering, pear fruit development depends mainly on cell division, after which the fruit size enlarges only through cell expansion accompanied by the accumulation of sugars (Bain, 1961; Yamaki & Matsuda, 1977). V-PPase is an important enzyme for pear fruit development because it has high activity during cell division, a stage in which the relative growth rate is also high, and has essential activity in cell expansion stage (Shiratake, Kanayama, Maeshima & Yamaki, 1997). In this study, we report the purification and characterization of V-PPase from pear fruit as a first step in understanding the role that this enzyme plays in fruit development.

2. Results and discussion

2.1. Solubilization of V-PPase by lysophosphatidylcholine

The following procedure was effective in solubilizing significant amount of the V-PPase from the tonoplast of pear fruit. First, peripheral proteins were removed from the tonoplast preparation by solubilization with 1% Triton X-100. Non-solubilized proteins were collected through ultracentrifugation, and V-PPase was solubilized from the remaining proteins with 0.5%

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0031-9422/98 $ - see front matter
PII: S0031-9422(98)00554-8

Table 1
Purification of vacuolar H^+-PPase from pear fruit

	Total protein (mg)	Total activity (μmol/h)	Yield (%)	Specific activity (μmol/h mg)	Purification factor (fold)
Tonoplast	14.8	782	100	52.8	1
Triton ppt	5.36	744	95.2	139	2.6
LPC sup	4.51	499	63.8	111	2.1
Mono Q	0.231	88.2	11.3	383	7.2
Superose 6	0.008	6.6	0.8	850	16

lysophosphatidylcholine (LPC) treatment. The specific activity increased 2.1-fold and 63.8% of the total activity was recovered after this differential solubilization (Table 1).

2.2. Purification of V-PPase by Mono Q chromatogram and Superose 6 gel filtration

Typical purification steps are shown in Table 1. When LPC-solubilized tonoplast proteins were loaded on Mono Q column and eluted by a linear gradient of 0 to 1 M KCl, V-PPase activity was obtained in fractions eluted with *ca.* 0.5 M KCl (Fig. 1). The specific activity was highest in fractions 28 and 29, resulting in an increase of 7.25-fold in activity. Thus, both fractions were used for further purification.

The elution profile from Superose 6 gel filtration is shown in Fig. 2A. Relatively high concentration of polyoxyethylene 8 lauryl ether ($C_{12}E_8$) and KCl were added to the HPLC buffer to obtain good resolution. Initially, 1% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) was used instead of 0.4% $C_{12}E_8$, assuming that CHAPS would be more suitable for gel filtration since its micelle size is comparatively small (6 kDa). However, the proteins aggregated in the gel and 50% of V-PPase activity was lost (data not shown). Thus, $C_{12}E_8$ was used as detergent in spite of its large micelle size (68 kDa). The specific activity of the peak fractions following gel filtration was 850 μmol h^{-1} per mg protein, an increase of 16-fold compared to that in the tonoplast preparation. The solubilized V-PPase appeared homogenous on SDS-PAGE after these two column chromatographies. The enzyme was estimated to constitute about 6% of total vacuolar membrane proteins in pear fruit, as calculated from the specific activity (Table 1). This value is more than the constitution rate (1%) of red beet root which is a similar sink organ as pear fruit (Rea & Poole, 1993). This indicates that V-PPase may contribute greatly to the pumping function of the vacuolar membrane of immature pear fruit.

2.3. Molecular mass of V-PPase by SDS-PAGE and immunoblotting

SDS-PAGE of active fractions separated by Superose 6 revealed a polypeptide of 66 kDa whose concentration profile corresponded to the profile of ac-

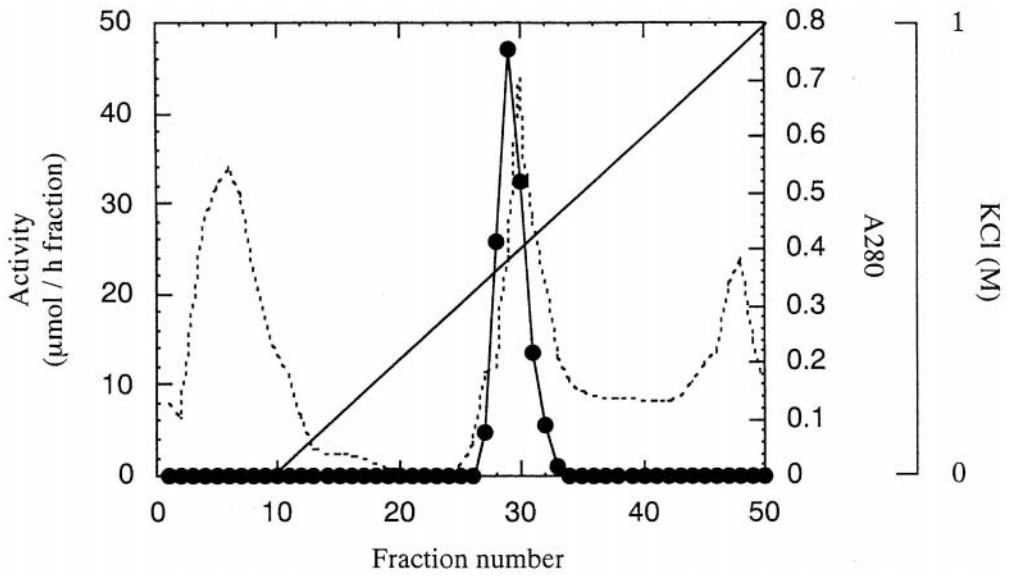

Fig. 1. Elution profile of V-PPase during Mono Q chromatography. V-PPase activity (●), protein (– – –), KC1 gradient (——).

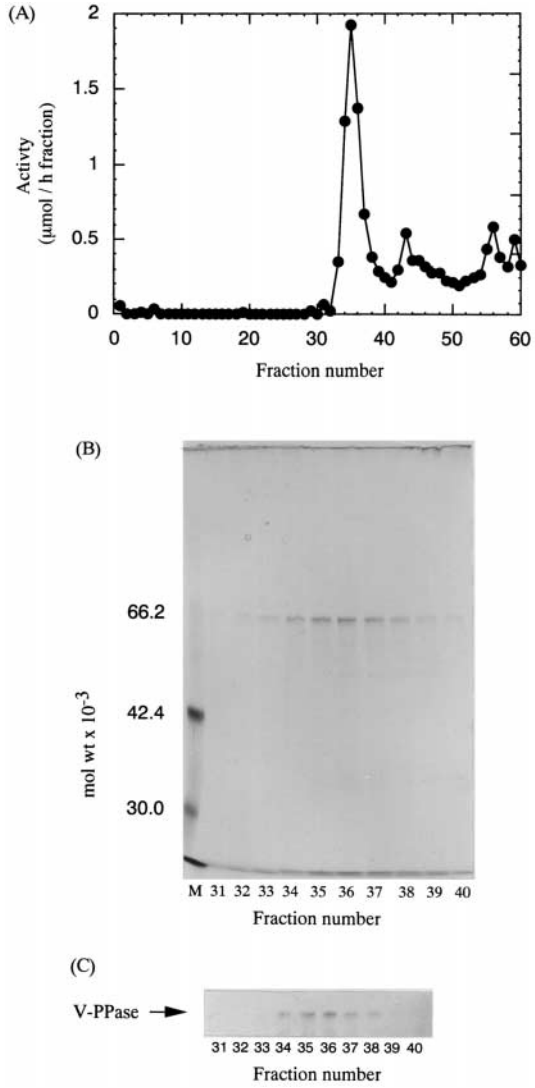

Fig. 2. Elution profile of V-PPase during Superose 6 chromatography (A) and SDS-PAGE (B), and distribution of immunoreactive polypeptide (C). Fractions 28 and 29 of Mono Q chromatography were loaded on Superose 6 column. For SDS-PAGE, 37.5 μl of each fraction was analyzed by 10.0% SDS-PAGE and silver-stained. For the Western blot, 12.5 μl of each fraction was subjected to SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was then immunostained with anti-mung bean V-PPase.

tivity described in Fig. 2A (Fig. 2B). The single band on SDS-PAGE cross-reacted with an antiserum against V-PPase of mung bean (Fig. 2C). The apparent *Mr* of V-PPase/detergent micelle was estimated to be 173 kDa by Superose 6 gel filtration. Considering that the *Mr* of V-PPase is 66 kDa, it is possible that each V-PPase/detergent micelle contains two V-PPase polypeptides and the difference of 41 kDa was derived from detergent micelle. Thus, V-PPase in pear fruit may be a homodimer of 66 kDa subunits as suggested by Sato, Maeshima, Ohsumi and Yoshida (1991).

2.4. Properties of V-PPase

The optimum pH of the purified V-PPase was 7.0–7.5 and the addition of 50 mM potassium stimulated the activity 4-fold (data not shown). *N*,*N*′-dicyclohexylcarbodiimide (DCCD) is an inhibitor of H^+-ATPase and H^+-PPase through blocking proton conductance (Sze et al., 1992; Maeshima & Yoshida, 1989). Therefore, the effect of DCCD on V-PPase activity was investigated to determine if V-PPase from pear fruit also has a proton channel. As shown in Fig. 3, more than 90% inhibition was observed at 1.5 mM DCCD, consistent with the present PPase being a H^+-PPase. Calcium is a specific inhibitor of V-PPase, unlike other H^+-translocases, such as V-, P-, and F-ATPases. In this experiment, the calcium concentration for 50% and more than 90% inhibition was 50 μM and 300 μM, respectively (Fig. 4), consistent with the inhibitory effect of calcium ion on V-PPase described by Maeshima (1991).

3. Experimental

3.1. Plant materials

Pear (*Pyrus communis* L. var. *sativa* DC, cv. La France) fruits were harvested in early May (about 40 days after full bloom) and stored at −1° in a polyethylene bag having some tiny pores.

3.2. Preparation of tonoplast vesicles

Tonoplast vesicles were isolated by the methods described previously (Shiratake, Kanayama & Yamaki, 1997). The flesh tissue of pear fruit (350 g) was homogenized at 4° with pestle and mortal in 1.75 l of 100 mM Tris/HCl buffer (pH 8.0) containing 250 mM mannitol, 2 mM EGTA, 0.5% (w/v) BSA (fraction V powder), 4 mM dithiothreitol (DTT), 5% (w/v) PVP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM $MgSO_4$. Further, the homogenate was homogenized with a glass homogenizer. The homogenate was centrifuged at 10,000*g* for 20 min. The supernatant was centrifuged at 100,000*g* for 30 min. The ppt was suspended in 140 ml of the suspension medium A (10 mM Bis-Tris-Propane (BTP)/HCl (pH 7.5), 250 mM mannitol, 1 mM DTT and 2 mM $MgSO_4$) and sonicated for 20 s. After the suspension was centrifuged at 10,000*g* for 20 min, the supernatant was centrifuged at 100,000*g* for 30 min. This microsomal ppt was suspended in 140 ml of 10 mM BTP/HCl (pH 7.5) buffer containing 500 mM sucrose, 1 mM DTT and 2 mM $MgSO_4$ and poured into centrifuge tube. The suspension was overlayered with the suspension medium A. After centrifugation at 100,000*g* for 1 h with

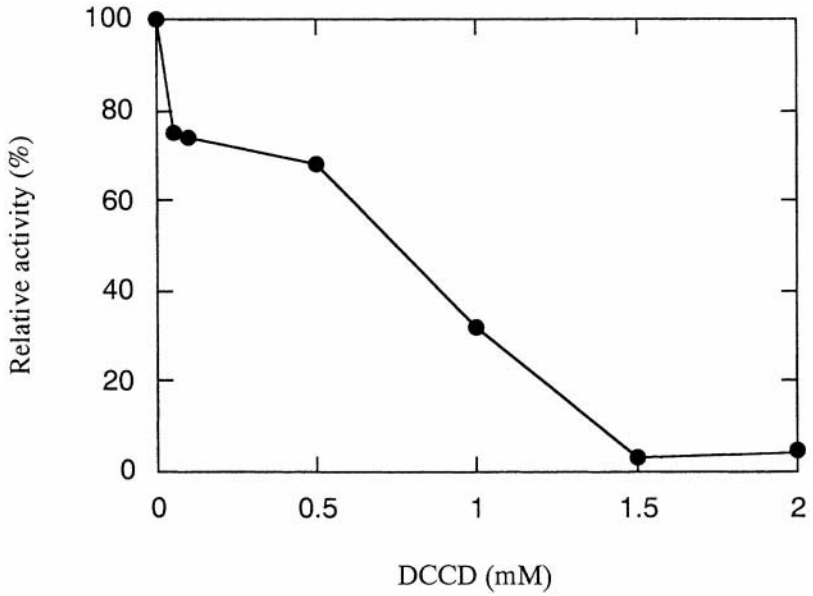

Fig. 3. Effect of DCCD on purified V-PPase activity. The V-PPase activity was assayed in the presence of various concentration of DCCD as described in Experimental.

RPS27-2 rotor (Hitachi, Tokyo, Japan), the interface layer was collected and diluted with the suspension medium A. The suspension was centrifuged at 125,000*g* for 30 min. The ppt was suspended with the suspension medium B (20 mM BTP/HCl (pH 7.5), 1 mM EGTA, 4 mM $MgSO_4$, 1 mM DTT), and 20% (v/v) glycerol), and protein concn adjusted to *ca* 4 mg ml^{-1}. The suspension was used as the tonoplast preparation and stored at −80° until used.

3.3. Solubilization of V-PPase from the tonoplast preparation

An equal volume of 2% (v/v) Triton X-100 soln was added to the tonoplast preparation and incubated at 4° for 15 min. The sample was diluted with suspension medium B without glycerol until the centrifuge tubes were full, and centrifuged at 200,000*g* for 1 h. The ppt was suspended in suspension medium B at a concn of

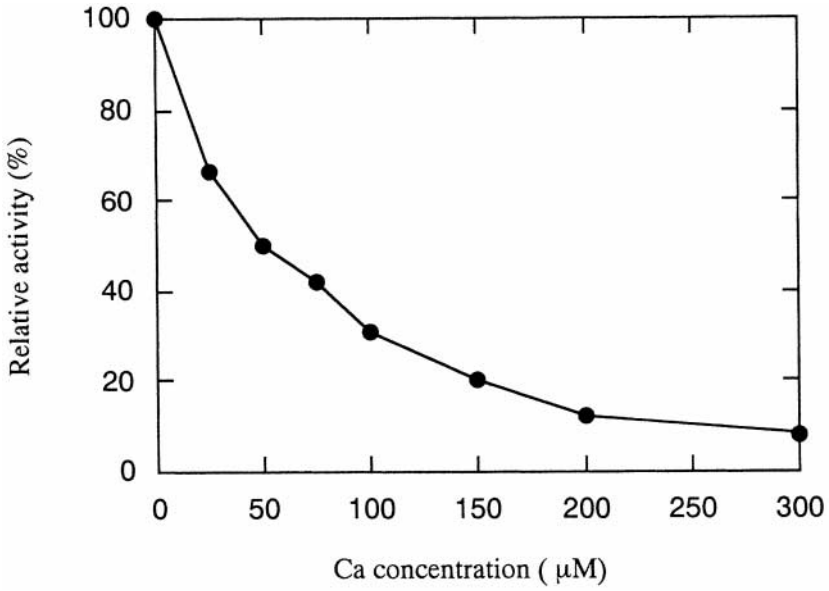

Fig. 4. Effect of calcium on purified V-PPase activity. The V-PPase activity was assayed in the presence of various concentration of calcium chloride.

ca 3 mg protein ml^{-1}. An equal volume of suspension medium B containing 1% (w/v) LPC (Egg yolk, Type I, Sigma) soln was added to the sample. The mixture was incubated at 30° for 30 min and centrifuged at 100,000*g* for 1 h. The supernatant was used as solubilized V-PPase.

3.4. Purification of V-PPase

The solubilized fraction of V-PPase was applied to a column of Mono Q HR5/5 (Pharmacia) pre-equilibrated with HPLC buffer (20 mM BTP/HCl (pH 7.5), 10% (v/v) glycerol, 4 mM $MgSO_4$, 1 mM DTT, 0.05 mg/ml L-α-phosphatidylcholine (Soybean, Type IV-S, Sigma), and 0.4% (w/v) $C_{12}E_8$). The column was washed with 10 ml HPLC buffer and the absorbed proteins were eluted with a linear gradient of 0 to 1 M KCl in HPLC buffer (Flow rate: 0.25 ml/min; Fraction: 0.5 ml). The fractions which had highest specific activity were pooled, concentrated to 200 μl with USY-1 (Advantec), and loaded on Superose 6 HR 10/30 (Pharmacia) preequilibrated with HPLC buffer containing 1 mM EGTA (omitted when testing effects of inhibitors) and 400 mM KCl (Flow rate: 0.1 ml/min; Fractions: 0.4 ml).

3.5. Polyacrylamide gel electrophoresis

SDS-PAGE was conducted using a 10.0% acrylamide gel according to Laemmli (1970).

3.6. Protein blotting

Proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane. The membrane was stained by the antibody against mung bean V-PPase (provided by Dr. M. Maeshima, Nagoya University). Goat anti-rabbit IgG conjugated with alkaline phosphatase was used as the second antibody.

3.7. Protein determination

Protein content was determined by a modification of the method of Bensadoun and Weinstein (1976). BSA was used as a standard.

3.8. Assay

PPase activity was assayed by modification of the method of Maeshima and Yoshida (1989). The reaction mixture for V-PPase contained 1 mM sodium PPi, 1 mM $MgSO_4$, 50 mM KCl, 1 mM sodium molybdate, 0.02% Triton X-100, 66 g/ml L-α-phosphatidylcholine and 50 mM BTP/2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 7.5). The enzyme activity of V-PPase was colorimetrically determined by measuring the rates of liberation of Pi at 30°. When testing the effect of DCCD on V-PPase activity, it was dissolved in EtOH. EtOH was added to each reaction mixture to the same volume of DCCD soln. The volume of EtOH did not affect the activity.

Acknowledgements

The authors thank Dr D. Maeshima, Nagoya Univertsity, Japan for the kind gift of mung bean V-PPase antibodies.

References

Bain, J. M. (1961). *Aust. J. Bot., 9,* 99.
Becker, A., Canut, H., Luttge, U., Maeshima, M., Marigo, G., & Ratajczak, R. (1995). *J. Plant Physiol., 146,* 88.
Bensadoun, A., & Weinstein, D. (1976). *Anal. Biochem., 701,* 241.
Britten, C. J., Turner, J. C., & Rea, P. A. (1989). *FEBS lett., 256,* 200.
Hosaka, M., Kanayama, Y., Shiratake, K., & Yamaki, S. (1994). *Phytochemistry, 36,* 565.
Laemmli, U. K. (1970). *Nature, 227,* 680.
Maeshima, M. (1991). *Eur. J. Biochem., 196,* 11.
Maeshima, M., Miura, T., & Sato, T. (1994). *Plant Cell Physiol., 35,* 323.
Maeshima, M., Nakanishi, Y., Matsura-Endo, C., & Tanaka, Y. (1996). *J. Plant Res., 109,* 119.
Maeshima, M., & Yoshida, S. (1989). *J. Biol. Chem., 264,* 20068.
Perotti, E., Gavin, O., Widmer, F., & Chanson, A. (1994). *Plant Sci., 103,* 25.
Rea, P. A., & Poole, R. J. (1993). *Annu. Rev. Plant Physiol. Plant Mol. Biol., 44,* 157.
Sarafian, V., & Poole, R. J. (1989). *Plant Physiol., 91,* 34.
Sato, M. H., Kasahara, M., Ishii, N., Homareda, H., Matsui, H., & Yoshida, M. (1994). *J. Biol. Chem., 269,* 6725.
Sato, M. H., Maeshima, M., Ohsumi, Y., & Yoshida, M. (1991). *FEBS Lett., 290,* 177.
Shiratake, K., Kanayama, Y., Maeshima, M., & Yamaki, S. (1997). *Plant Cell Physiol., 38,* 1039.
Shiratake, K., Kanayama, Y., & Yamaki, S. (1997). *Plant Cell Physiol., 38,* 910.
Sze, H., Ward, J. M., & Lai, S. (1992). *J. Bioenerg. Biomembr., 24,* 371.
Taiz, L. (1992). *J. Exp. Biol., 172,* 113.
Yamaki, S., & Matsuda, K. (1977). *Plant Cell Physiol., 18,* 81.