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Comparison of two systems of tonoplast purification from tobacco cells of suspension culture BY-2

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Abstract

The tonoplast is an intracellular membrane, important for numerous functions of the vacuoles in plant cells. Transport, signaling, enzymatic activity and other processes of the tonoplast are the focus of a wide spectrum of studies. Multiple advanced analyses demand highly purified vacuoles or vesicles of the tonoplast membrane fraction. Since 1960 several approaches have been developed for such purification, but new goals and new model objects require adjustment of already existing techniques. The presented investigation aimed to compare two methods of tonoplast membrane fraction purification from tobacco suspension cell culture BY-2 (*Nicotiana tabacum* L., cv Bright Yellow) based on sucrose and sucrose/sorbitol gradients. The functional activity of obtained purified tonoplast fractions was measured by the hydrolytic activity of vacuolar H^+-ATPase and H^+-PPase.

Keywords: tonoplast, vesicle fraction, purification, V-ATPase, H^+-PPase.

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Introduction

The tonoplast is an endogenous membrane surrounding the central vacuole which fulfills a crucial role in the biochemistry and physiology of a plant cell. It participates in the generation and maintenance of cell turgor. It is involved in elongation growth as well as different processes of autophagy and even cell death (Hatsugai et al., 2015; Kruger and Schumacher, 2018). Vacuoles vary significantly in number and size due to the cell type and developmental stage (Zhang, Hicks and Raikhel, 2014). Vacuoles participate in nutrition storage, detoxification, adaptation to salt stress and pathogen attack (Martinoia et al., 2007; Trentmann and Haferkamp, 2013). The origin of the plant cell central vacuole is still debated (Kruger and Schumacher, 2018). It might originate from the ER (Endoplasmic reticulum) subdomains and/or the *trans*-Golgi network (Mesquita, 1969; Marty, 1978). Central vacuole biogenesis is closely linked to actin-myosin cytoskeleton reorganization and is regulated by 1NAA (1-naphthylacetic acid), a synthetic auxin analogue; this indicates a possible mechanism involved in the elongation growth, triggered by auxin (Scheuring et al., 2016).

The tonoplast is enriched by different transporters. Special attention is always paid to tonoplast proton pumps. H^+-ATPase and H^+-PPase are known to use the energy of macroergic bonds for generation of the proton gradient important for secondary active transport of different ions and compounds (Dettmer et al., 2006; Schumacher, 2006). The direction of transport is important to provide pH and

Ca^{2+} cytosolic homeostasis and to equalize the osmotic potential between the vacuole and cytosol.

Investigation of tonoplast morphology and cross-interaction between the tonoplast and other intracellular membranes might be visualized with freeze-etching electron microscopy and 3D high-voltage electron microscopy. Tonoplast biochemical composition alteration during development or stress adaptation requires modern systemic approaches like proteomics (Trentmann and Haferkamp, 2013). Thus, correct vacuole preparation and the procedure of tonoplast membrane fraction enrichment acquire a special importance.

One of the first methods to obtain vacuoles by osmotic lysis from protoplasts was developed in the 1960s (Cocking, 1960). It was adjusted for vacuole preparation from different plant tissues and plant species (Gregory and Cocking, 1966; Wagner and Siegelman, 1975). Recently the method was corrected for gathering vacuoles from *Arabidopsis* leaves (Trentmann and Haferkamp, 2013). But some observations narrow the application of this method. The first one concerns the use of enzymes for protoplast isolation. The procedure requires three to five hours to digest the cell wall, so it is hardly applicable for the investigation of fast cell responses triggered by light signals, stress factors, etc. Another approach to obtain a reasonable yield of protoplasts is gentle mechanical disruption of the tissue (Leigh and Branton, 1976; Salyaev et al., 1981). Thin slicing is suitable for different plant tissues, including very robust ones such as a storage beetroot of *Beta vulgaris*. Further vacuole purification after osmotic lysis of protoplasts is usually provided by a gradient of sorbitol, ficoll, KCl, metrizamide, etc. The stability and viability of vacuoles depend significantly on the gradient nature and pH level. However, these substances used for osmotic lysis might cause alteration in the tonoplast enzyme activity and even loss of peripheral proteins. Hence, the purity of the vacuoles and tonoplast fraction obtained is high and can be used for further highly specific proteome analysis (Trentmann and Haferkamp, 2013).

Nevertheless, for the investigation of functional activity of the tonoplast, especially for the analysis of the activity of its enzymes and transporters, another method of purification was used widely. Tonoplast vesicles from homogenized plant tissues are purified by ultracentrifugation in a density gradient. According to a number of studies, membrane fractions enriched with tonoplast are supposed to be located between 14% and 26% sucrose layers (Leonard and Vanderwoude, 1976; Tankelyun et al, 1986; Briskin, Leonard and Hodges,1987; Larsson, Widell and Kjellbom, 1987; Tankelyun, 1998; Shakhova and Tankelyun, 2008). This method of purification was used to detect activity of different enzymes including tonoplast H^+-ATPase and proton PP-ase (Mettler and Leonard, 1979; Dupont, Bennett and Spanswick, 1982; Churchill and Sze, 1983). The sucrose-gradient centrifugation is not ideal to obtain a fraction free of endomembrane contamination, and thus it consistently was replaced by another approach. The problem was solved by the method of purification based on the sorbitol/sucrose gradient centrifugation (Maeshima and Yoshida, 1989; Maeshima, 2000).

Taken together, purified tonoplast/vacuole could be obtained by a few methods suitable for a wide spectrum of model objects represented by different plant organs and tissues. But still the method type has to be tested for each plant material and might require some adjustments. For example, in cells of suspension culture, because of the continuous shaking, the mechanical properties of the cell wall might differ from those of plant cells of native tissues; in this case the application of enzymatic or slicing methods would be rather questionable. The presented investigation is aimed to compare two methods of tonoplast membrane fraction purification from tobacco suspension cell culture based on sucrose and sucrose/sorbitol gradients.

Material and methods

Plant material. Etiolated suspension tobacco cell culture BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow) was used for tonoplast isolation. Initially this culture was obtained from tobacco leaf mesophyll cells and cultivated in modified Murashige-Skoog (MS) salt medium (Murashige and Skoog, 1962), containing 30 g l^{-1} (0.09 M) sucrose and 0.2mg l^{-1} 2,4-dichlorophenoxyacetic media (Kobayashi, Niino and Kobayashi, 2006). BY-2 cells in suspension show synchronized development and grow relatively slowly (18 days is the full life cycle), which makes this line attractive for different type of studies (Hasezawa, 1983; Nagata, Nemoto and Hasezawa, 1992; Nagata and Kumagai, 1999; Zazimalova, Petrášek and Morris 2003; Nagata, Hasezawa and Inze, 2004; Petrášek and Zazimalova, 2006). BY-2 suspension was cultivated in liquid media on an orbital shaker (110 rpm) at 25 °C in the darkness. Cells were transferred to fresh medium every 18 days. For tonoplast isolation 14-day-old cells were used at the stage of elongation growth (Petrášek and Zazimalova, 2006).

Sucrose-density gradient (SDG) method. This method was previously developed for maize coleoptiles (Tankelyun, 1998; Shakhova and Tankelyun, 2008). The main scheme for tonoplast enrichment is shown in Figure 1. All steps were conducted at 4 °C. We grinded 30 g of cells in a homogenizator Wisd23 HG-15D (5 min, 10,000 rpm) in 30 mL of SDG-I medium (0.4 M Sucrose, 50 mM Tris/MES pH 7.8, 5 mM Na_2EDTA, 0.03 M ascorbic acid, 2.5 mM DTT). We added 20 mL of SDG-1 medium to the homogenate and performed two-step centrifugation: 100 g for 5 min and 4200 g for 10 min

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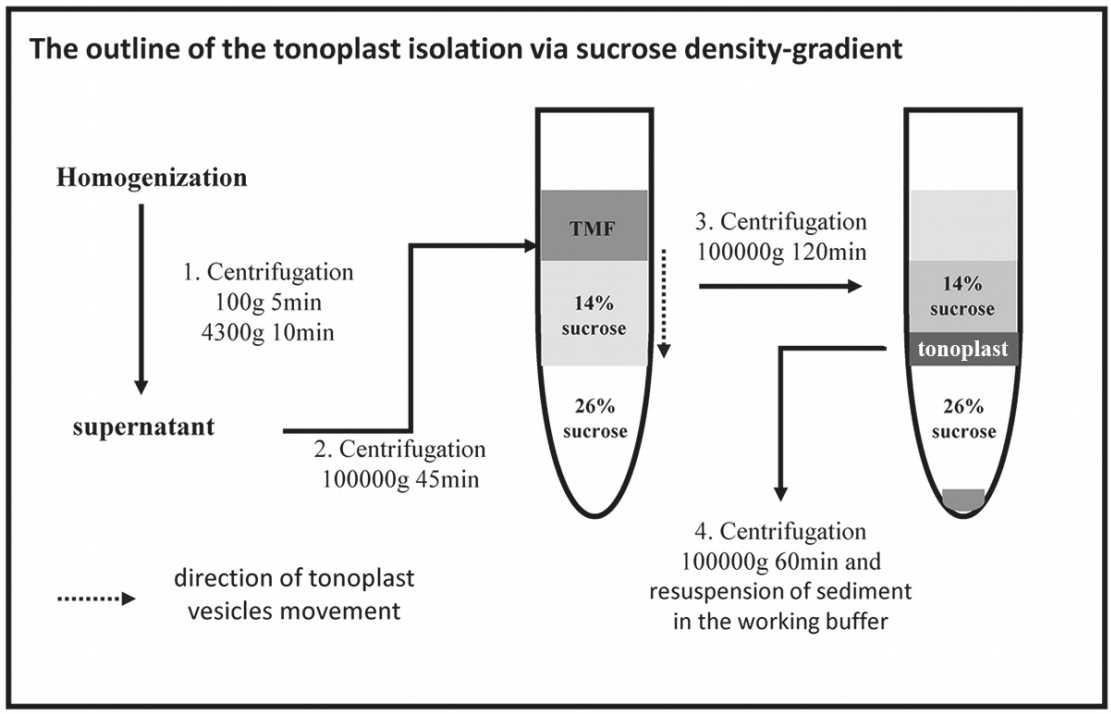

Fig. 1. The scheme of tonoplast isolation via the sucrose density gradient (SDG) method.

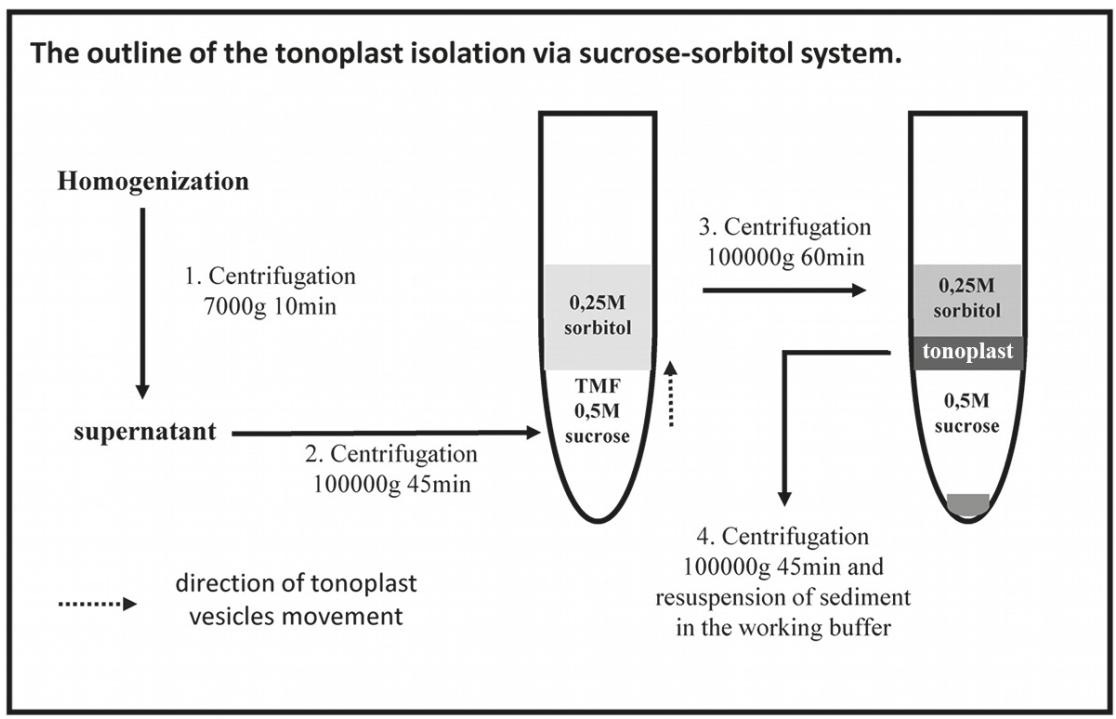

Fig. 2. The scheme of tonoplast isolation via the sucrose-sorbitol system (SSS) method.

(MPW-350R centrifuge). The sediment, containing unbroken cells, nuclei, mitochondria and plastids, was discarded. The supernatant of accumulated cell membranes was centrifuged at 100,000g for 45 min (ultracentrifuge Beckman Avanti J-30I). The obtained sediment, consisting of total microsomal fraction (TMF), was resuspended in medium SDG-II (0.2 M sucrose 5 mM Tris-Mes pH 7.2, 1 mM Na_2EDTA, 1 mM DTT) in a Potter-Elvehjem homogenizator. For the gradient, 14 % and 26 % sucrose solutions prepared in 1 mM Tris-Mes buffer pH 7.2 were gently layered and then 1 mL of resuspended TMF was added on the top. After centrifugation (100,000g for 2 h)

the targeted tonoplast-enriched fraction (TEF) was collected on the border of the sucrose solutions. Next, the TEF was resuspended in medium SDG-II and centrifuged again at 100,000g for 60 min. The obtained sediment contained the purified TEF. The purified TEF was suspended in medium SDG-II and stored at –80 °C.

Sucrose-sorbitol system (SSS) method. Initially the SSS method was developed by Maeshima and Yoshida (1989) for mung bean hypocotyl tissues. In this investigation it was adjusted for tobacco cell suspension culture. The scheme of tonoplast-enrichment vesicle fraction purification by the sucrose-sorbitol method is shown in Figure 2. All steps were carried out at 4 °C. Cells (30 g) were grinded with a homogenizer Wisd23 HG-15D (5 min, 10,000 rpm) in 30 mL of medium SSS-I (0.25 M Sorbitol, 50 mM Tris/MES pH 7.5, 1 mM EGTA, 2 mM DTT, 1 % (W/V) PVP, 0.2 % (W/V) BSA). The homogenate was centrifuged 10 min at 7000g (MPW-350R centrifuge). The sediment was discarded and the supernatant was centrifuged at 100,000g for 45 min (ultracentrifuge Beckman Avanti J-30I). The sediment, containing TMF, was collected in medium SSS-II (0.5 M sucrose, 20 mM Tris/MES pH 7.5, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT) and transferred to a Potter-Elvehjem homogenizer. Resuspended TMF was applied on the top of sorbitol medium SSS-III (0.25 M sorbitol, 20 mM Tris/MES pH 7.5, 1 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT). This sucrose-sorbitol system was centrifuged at 100,000g for 60 min. The vesicle fraction enriched by tonoplast (TEF) was collected at the border between sucrose and sorbitol. It was then diluted with medium SSS-III and centrifuged at 100,000g for 45 min. The sediment was resuspended in SSS-III and frozen at –80 °C until further analysis.

Protein Determination. For a quantitative protein assay M. Bradford's (1976) micromethod was employed. This method is based on specific sorption of Coomassie brilliant blue G-250 dye by proteins. A protein sample was added to a solution of the dye in phosphoric acid and ethanol. Extinction was measured at 595 nm on a Spekol-1300 spectrophotometer (Analytic Jena). For a calibration curve we used the ovalbumin solution in 150 mM sucrose and 10 mM Tris–MES pH 7.2.

ATPase and PPase assays. The hydrolytic activity of membrane H^+-ATPase or H^+-pyrophosphatase was measured by the level of inorganic phosphorus (Pi) resulting from enzymatic hydrolysis of ATP or inorganic pyrophosphate (PPi), respectively. Hydrolytic activity was tested at the appropriate optimal pH: 6.0 for plasma membrane ATPase; 7.2 for tonoplast H^+-ATPase and H^+-pyrophosphatase (Briskin, 1991).

A hydrolytic reaction was started by adding 25 µL of membrane fraction to 125 µl of a reaction mixture containing 90 mM Tris-Mes, 15 mM ATP or sodium pyrophosphate, 0.25 M KCl, 15 mM $MgCl_2$. Eppendorf microtubes with the reaction mixture were incubated for 30 min at 37° C in a water thermoshaker. For nonenzyme ATP hydrolysis, test reaction mixture without KCl and $MgCl_2$ was used. K^+ and Mg^{2+} ions are mandatory for membrane H^+-ATPase activity. The hydrolytic reaction was stopped by adding 50 µl of 20 % trichloracetic acid and cooling in an ice bath.

Inorganic phosphorus in the reaction mixture was determined spectrophotometrically (Lindeman, 1958). The method is based on the formation of the phosphomolybdic acid complex, which becomes colored by a reducer ($SnCl_2$). Staining developed for 30 min and was measured at 750 nm on Spekol-1300.

Inhibitor analysis. For the determination of possible contamination of the tonoplast vesicle fraction with plasma membrane, further inhibitors were used: Na_3VO_4 (sodium orthovanadate) — a specific inhibitor of H^+-ATPase plasmalemma (pH 6.0) — and bafilomycin — an inhibitor of tonoplast H^+-ATPase (pH 7.2).

Statistics. All experiments were done in at least three biological and three analytic replicates. Standard deviation was calculated using the STDEV function in the package STDEV. S (Std Dev, Standard Deviation). Statistical significance was calculated using GraphPad Prism 7 software. Average values and error of mean are shown in the diagrams. Significance of the differences was calculated with ANOVA (One-way analysis of variance). Statistically significant differences ($P \leq 0.05$) are marked as*; Values of $P \leq 0.01$ of statistical significance are marked as**.

Results and discussion

The results of modern proteome analysis revealed the importance of the vacuole/tonoplast isolation, which affects the quality and quantity of identified proteins (Trentmann and Haferkamp, 2013). It is no less important to obtain a highly purified fraction for the analysis of tonoplast activity.

Two methods developed for tonoplast purification were compared in this investigation. The first one is based on tonoplast vesicle separation from other plant cell membranes according to their density. According to literature data this parameter varies as follows: tonoplast, 1.10–1.12 g/cm^3, Golgi membranes, 1.12–1.15 g/cm^3, rough endoplasmic reticulum, 1.15–1.17 g/cm^3, thylakoids, 1.16–1.18 g/cm^3, plasma membrane, 1.14–1.17 g/cm^3, and mitochondrial membranes, 1.18–1.20 g/cm^3 (Leonard and Vanderwoude, 1976; Briskin, Leonard and Hodges, 1987; Larsson, Widell and Kjellbom, 1987).

After grinding, tobacco cell homogenate was freed of unbroken cells, nuclei, mitochondria and plastids by low speed centrifugation (Fig. 1). The collected supernatant was used to sediment total microsomal fraction. The tonoplast was separated from the TMF with the

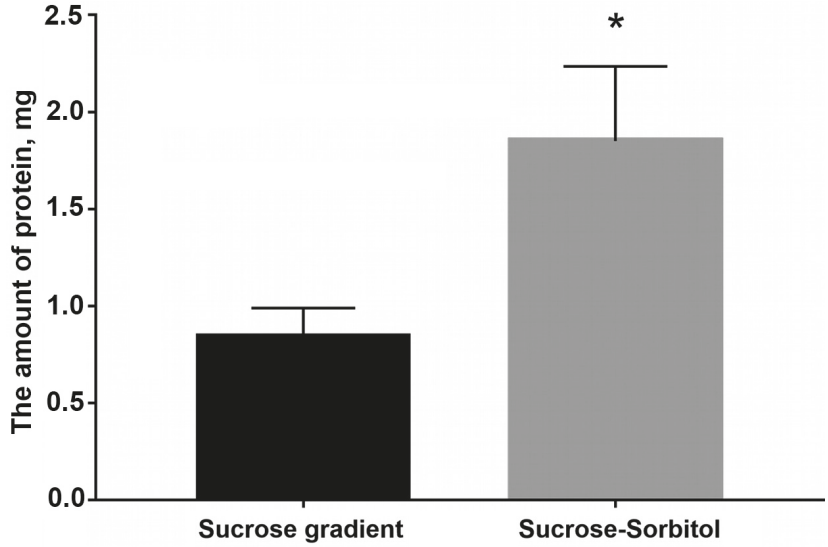

Fig. 3. The amount of proteins in tonoplast-enriched fractions obtained by SDG and SSS methods.

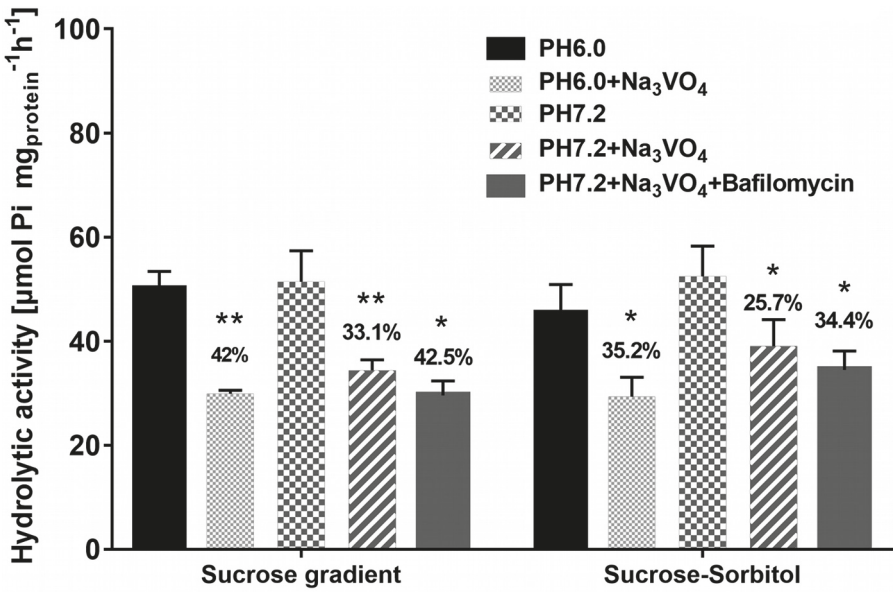

Fig. 4. Effects of pH and inhibitors on ATPase activity of total microsomal fractions obtained as parts of SDG and SSS methods.

sucrose gradient on the border between 14% and 26% sucrose solutions. The TEF was concentrated by ultracentrifugation and used for further analysis.

Alternatively, tobacco cells were homogenized and prepared as shown in Figure 2 until TMF. In this approach the microsomal fraction was separated via the sucrose/sorbitol system. The tonoplast vesicles accumulated on the border between sorbitol and sucrose layers. As in the SDG method, the tonoplast fraction was sedimented by ultracentrifugation and used for comparison.

Firstly, both SDG and SSS tonoplast fractions were used for a protein assay. As shown in Figure 3 the SSS method gave 55% greater protein accumulation. This higher protein yield positively characterized the sucrose/sorbitol system.

Further analysis focused on the quality of tonoplast fractions, i.e., the degree of purification. Fraction quality was estimated by inhibitor analysis, based on the activity of marker enzymes. The plasma membrane usually is the main contaminant of a tonoplast fraction. The marker enzyme of the plasma membrane is P-type H^+-ATPase with optimum pH 6.0, inhibited by vanadate (Na_3VO_4). Vanadate ions selectively bind in the active center with a carboxylic group of aspartic acid residue instead of phosphorus (Boldyrev, 1990; Wach, Ahlers and Gräber, 1990). Vacuolar V-type H^+-ATPase is con-

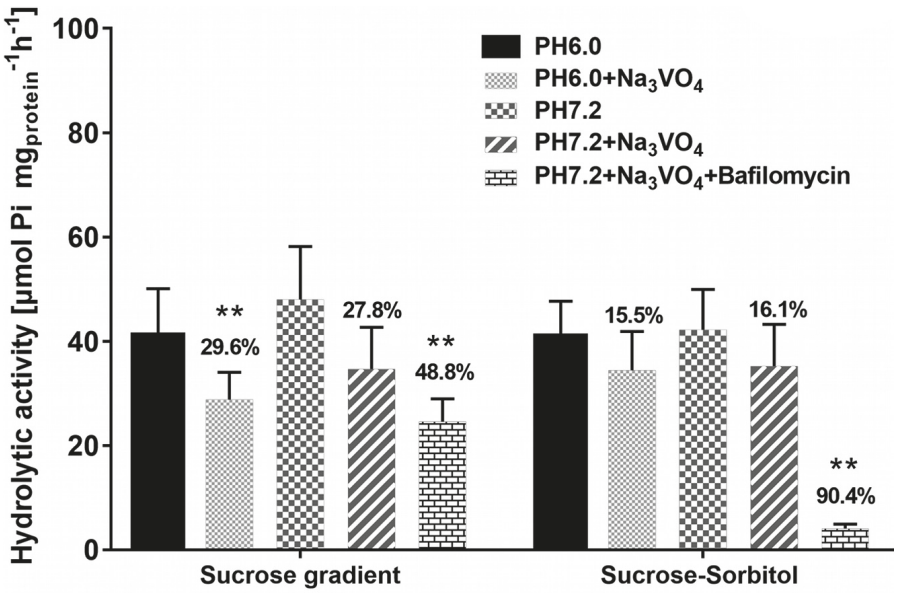

Fig. 5. Effects of pH and inhibitors on ATPase activity of tonoplast-enriched fractions obtained by SDG and SSS methods.

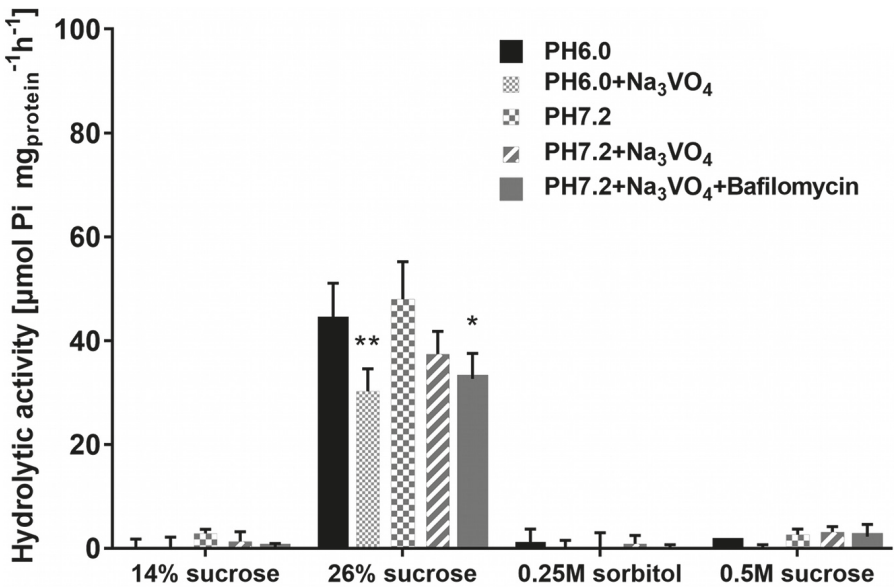

Fig. 6. Effects of pH and inhibitors on ATPase activity of vesicles obtained from working solution using two different methods of tonoplast enrichment. The SDG method used 14 % and 26 % sucrose solution. The SSS method used 0.25M sorbitol and 0.5M sucrose solution.

sidered a marker enzyme of the tonoplast. The multisubunit protein complex has optimum pH of 7.2. The selective inhibitor of a V-type tonoplast H^+-ATPase is bafilomycin (Ohkuma et al., 1993). In case of implication of both enzymes for fraction characterization, the determination of Pi accumulation as a result of ATPase activity at different pH and with the presence of inhibitors would indicate a possible contamination of the tonoplast fraction.

Initially H^+-ATPase activity was tested in total microsomes obtained from tobacco cells, and it later was used for purification. Figure 4 presented data on a hydrolytic assay of TMF. In collected microsomal membranes P-type and V-type H^+-ATPase activities were detected. Vanadate and bafilomycin were effective at inhibiting Pi release. Mean values were similar in both of the schemes used.

The hydrolytic activity of the tonoplast fractions obtained by the two different methods was quite similar, at about 40 moles Pi per mg of protein in 1 h (Fig. 5). Vanadate decreased the ATPase activity of SDG-obtained TEF by 29.6 % (pH 6.0) and 27.8 % (pH 7.2) (Fig. 5). SSS-

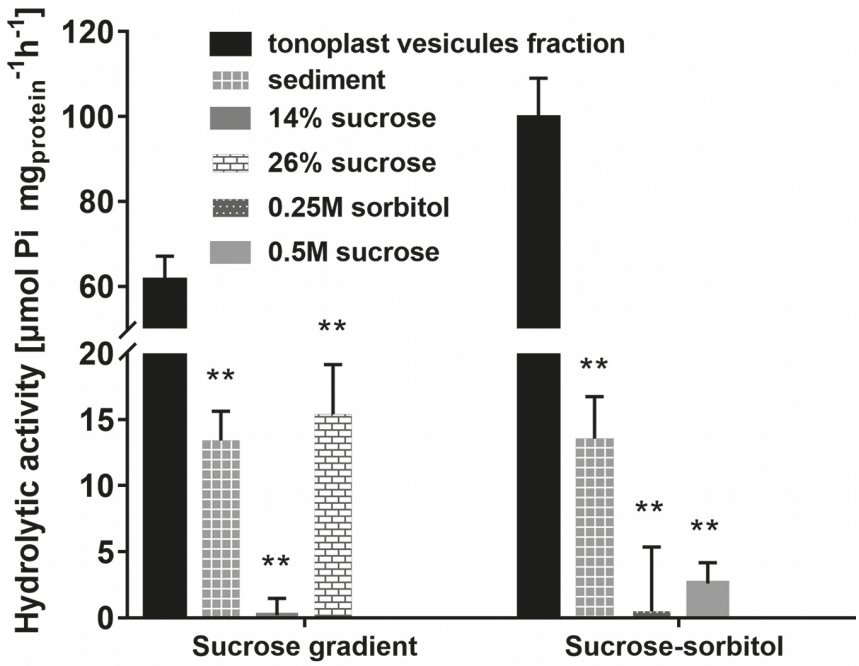

Fig. 7. Pyrophosphatase activity of tonoplast-enriched fractions and vesicles from working solutions and sediments, obtained as parts of SDG and SSS methods.

obtained TEF was almost half as sensitive to vanadate: by 15.5 % and 16.1 % at pH 6.0 and 7.2, respectively, but differences were not statistically significant. This proves that plasma membrane contamination in TEF obtained via the SSS method is minor. Bafilomycin, known as a highly selective inhibitor of V-type H^+-ATPases, decreased fraction hydrolytic activity by 21 % in SDG-TEF and by 74.3 % in SSS-TEF. Thus after sucrose/sorbitol purification, the vesicle fraction was highly enriched with tonoplast.

Subsequent analysis concerned detection of residual H^+-ATPase activity in sucrose solutions with the SDG method and sucrose/sorbitol media with the SSS method after gathering the tonoplast fraction. Presented data (Fig. 6) revealed that sucrose/sorbitol media were free of any membrane contamination, which would demonstrate H^+-ATPase activity. The same absence of activity was estimated for the 14 % sucrose solution. Accumulation of Pi was detected only in the 26 % sucrose layer. Application of vanadate and bafilomycin caused inhibition. Thus this part of the gradient is characterized by leftovers of plasmalemma and tonoplast. According to obtained data, the sucrose/sorbitol scheme is optimal to decrease the loss of tonoplast.

An additional enzyme that could be suggested as a marker for tonoplast is H^+-pyrophosphatase. Determination of its activity is presented in Figure 7. TEFs showed maximal activity, but it was higher with the SSS method. The sucrose gradient approach was characterized by half amplitude activity in the 26 % sucrose layer, which was shown to be contaminated by plasma membrane and tonoplast, according to the hydrolytic test (Fig. 6).

Conclusion

Obtained data indicated that the sucrose/sorbitol method is preferable for purification of the tonoplast fraction from tobacco suspension cell culture. This method turned out to be better in terms of the vacuolar membrane fraction quality, and less time-consuming as well. The tonoplast vesicle fraction was suitable for the investigation of the activity of both vacuolar proton pumps: H^+-ATPase and H^+-pyrophosphatase. Marker enzymes revealed sensitivity to specific inhibitors and optimum pH value.

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