Isolation of Vacuoles from the Upper Epidermis of *Petunia Hybrida* Petals
II. Vacuolar Localization of Some Hydrolases

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In vacuoles isolated from petal upper epidermis of *Petunia hybrida* through polybase-induced lysis of protoplasts the vacuolar localization of α-mannosidase and α-galactosidase was demonstrated. No association was observed between the tonoplast and these hydrolases. Protoplasts from different mutant lines blocked in anthocyanin biosynthesis were treated with DEAE-dextran to release vacuoles. The differences in quality of the obtained vacuolar preparations indicated the relation between mutant line used and successful application of the polybase-procedure.

Introduction

The research on the localization of hydrolases in plant cells has been strongly stimulated through the proposal of Matile [1], that the plant vacuole is analogous to animal lysosomes.

Consequently, the presence of various hydrolytic enzymes in plant vacuoles has been demonstrated. In some reports α-mannosidase has been proposed as a vacuolar marker enzyme [2, 3].

Previously we used anthocyanin as a vacuolar marker to develop a procedure for the isolation of naked vacuoles from petals of the coloured line *R27* of *Petunia hybrida*. For the characterization of vacuolar preparations from mutants which lack anthocyanin biosynthesis it became inevitable to search for a general vacuolar marker enzyme for protoplasts obtained from upper epidermis of *Petunia* petals.

We therefore investigated the vacuolar localization of some hydrolases to determine whether any of these enzymes can serve as a vacuolar marker. At our institute a variety of inbred lines of *Petunia hybrida* is present. The polybase procedure, resulting in good vacuolar preparations from the line R27 as previously described, was applied to other lines. The lines W78, W22, W80 and W98 were used. These lines are recessive for the genes *An1*, *An2*, *An6* and *An9*, respectively (Table I). Mutations on the *An*-genes affect the terminal steps of anthocyanin biosynthesis in the epidermis of petals. Mutants recessive for one or more of the *An*-genes possess white flowers or faintly coloured flowers in the case of a mutant recessive for the *An2* gene only. The lines R27 and W28 have an isogenic background: the line W78 is derived from the line R27 and differs only with respect to the *An1* gene. The lines W22, W80 and W98 have different genetic backgrounds.

Abbreviations: PNP, *p*-nitrophenyl; DEAE, diethylaminoethyl; MES, (2-[N-morpholino]ethane-sulphonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid; PVP, polyvinyl pyrrolidone; BSA, bovine serum albumin; G6PDH, glucose-6-phosphate dehydrogenase; PEG, poly-ethylene-glycol.

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Materials and Methods

Plant material

Petunia hybrida R27, W22, W78, W80 and W98 were cultivated in a growth chamber under 16 h day (21 000 Lux) at 21 °C. The plants were fertilized bimonthly with Pokon 16:21:27 and Sequestrene 138 Fe and watered each day.

Chemicals

Cellulase Onozuka *R10* and Macerozyme Onozuka *R10* were purchased from Kiuki Yakult (Nishinomiya, Japan); DEAE-dextran and Ficoll 400 were obtained from Pharmacia (Uppsala, Sweden). Dextran-sulphate, Mes, Hepes, PEG6000, BSA and D-mannitol from Sigma (St. Louis, USA).

Assays

Anthocyanin was extracted and measured as described by Jonsson *et al.* [4]. Protein was determined according to Bradford (5). Glucose-6-phosphate dehydrogenase was assayed spectrophotometrically by monitoring changes in optical density at 340 nm [6]. Peroxidase was assayed according to Van den Berg *et al.* [7]. Hydrolase activities were measured spectrophotometrically at 415 nm by recording the release of PNP from artificial PNP-substrates. The assay medium (0.2 ml) contained besides sample an excess PNP-substrate and 0.25 M acetate buffer pH 5.0. The assay was performed at 30 °C under shaking, stopped by addition of 0.8 ml ice cold 0.3 M NaOH-glycine pH 10.6 and clearified by centrifugation at 10000 × *g* during 5 min.

Prior to enzyme assays protoplast and vacuolar fractions were lysed sonically, while suspended in 0.6 M mannitol. The lysate of protoplast fractions was diluted with 0.6 M mannitol to an identical anthocyanin concentration as present in the corresponding vacuolar lysates.

It was achieved in this way that the concentration of vacuolar components was identical in the enzyme assays performed with vacuolar and corresponding protoplast preparations. Enzyme activities measured in these preparations in this way can be directly compared, which was the main goal of the assays. For this reason vacuolar and protoplast fractions were not dialyzed or treated with Dowex, PVP or PolyclarAT, in order to obtain enzyme preparations free of salts, phenolics and flavonoids, because it was observed that loss of enzyme activities occurred due to such manipulations.

Upper epidermis homogenate

Stripped tissue was grounded in a mortar in ice cold 0.1 M potassiumphosphate buffer pH 7.5 containing 1 mM mercaptoethanol. The homogenate was filtrated through a 50 µm nylon gauze and centrifugated during 5 min at 1600 × *g*. The supernate was collected and used for enzyme assays.

Preparation of protoplasts and vacuoles

Protoplasts were prepared from stripped upper epidermis of petals from different lines as described for the line R27 [8]. Because protoplasts derived from the lines W22 and W98 show a completely different buoyancy in a discontinuous Ficoll-gradient compared to protoplasts from the lines R27, W78 and W80 the polybase-gradient had to be adapted for W22 and W98 protoplasts. These protoplasts were suspended in 0.6 M mannitol and subsequently centrifugated downwards a Ficoll gradient, thereby passing the DEAE-dextran and dextran-SO_4. The polybase gradient used for W78 and W80 protoplasts was identical to the described gradient for R27 protoplasts [8].

Results

Vacuoles can be released from protoplasts of the lines W22, W78, W80 and W98 through application of the polybase procedure. In all cases the released vacuoles show the same buoyancy as the original protoplasts. The purity of final vacuolar preparations from W78 and W22 protoplasts was based on microscopical and biochemical examination comparable to the purity of vacuolar fractions from the line R27: 10–20% contamination. However, the yields were lower: less than 15%. Application of the polybase procedure on W80 and W98 protoplasts was less successful: up to 30% contamination with protoplasts and yields mostly less than 5%. Apparently, protoplasts derived from an identical tissue but from different lines of *Petunia hybrida* are differently sensitive for polybase-induced disruption of their plasmalemma.

Table I shows photomicrographs of protoplasts and isolated vacuoles from *An*-recessive lines. It can be seen from these micrographs that protoplasts and vacuoles have a diameter similar to protoplasts and vacuoles from the line R27 when suspended in the same medium. The extreme differences in buoyancy between protoplasts or vacuoles from different lines can therefore not be explained by differences in size. Table I shows that there exist no correlation between anthocyanin content and buoyancy in Ficoll-gradients. On the other hand, there is a suggestive correlation between buoyancy and vacuolar presence of flavonols. The lines W22 and W98 are dominant for the gene FL, resulting in accumulation of large amounts of flavonols in their epidermal vacuoles. Contrary, the lines R27, W78 and W80 are recessive for the gene FL and therefore accumulate only minor amounts of flavonols.

Hydrolase activities were measured in protoplast and vacuolar preparations and upper epidermis homogenate from the line R27. The hydrolase con-

tent of medium used for the preparation of protoplasts was also determined. Table II shows the results of these measurements.

A comparison of enzyme contents of protoplast preparation, upper epidermis homogenate and cellulase-macerozyme medium indicates that isolated protoplasts are not seriously contaminated with cell wall digesting medium. The hydrolase activities present in the protoplast preparation are also present in the homogenate and are therefore not caused by contamination. The differences in hydrolase composition of protoplast preparation and cellulase-macerozyme medium also point to the absence of contamination in the protoplast fraction.

A comparison of enzyme contents of protoplast preparation and homogenate suggests that peroxidase and β-glucosidase are mainly located extracellularly. A predominant cell wall localization of peroxidase and β-glucosidase is in agreement with prior observations at our laboratory. Hendriks, personal communication, found that peroxidase *c*, the major peroxidase isoenzyme in flower corolla tissue, is located extracellularly. In fact, vacuum infiltration of flower corolla tube releases most per-

Table I. Protoplasts and vacuoles released from upper epidermis of *Petunia* mutants (for specific information on mutations Ref. 14). +: homozygous dominant; −: homozygous recessive.

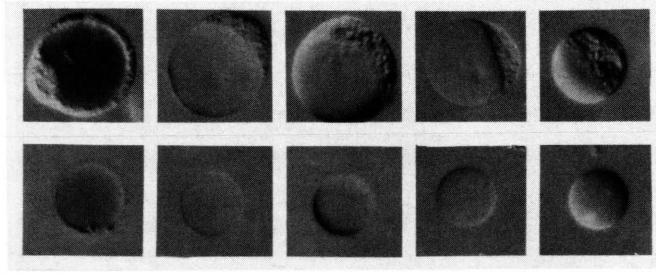
Line	R27	W78	W22	W80	W98
Protoplast (1:1500)					
Vacuole (1:1000)					
Genotype					
An1	+	−	+	+	+
An2	+	+	−	+	+
An6	+	+	+	−	+
An9	+	+	+	+	−
FL	−	−	+	−	+
Buoyancy in gradient (%)	3–6	3–6	12–17	3–6	12–17

Table II. Specific activities of some hydrolases in hydrolytic enzyme mixture, upper epidermis homogenate, protoplast and vacuolar preparations from the line R27, expressed as nmol/mg prfotein · min and as nmol/μmol anthocyanin · min (between parentheses), and relative enzyme activities in vacuoles based on anthocyanin as vacuolar marker.

Activity	Incubation mixture	Epidermis homogenate	Protoplast preparation	Vacuolar preparation	% in vacuolar preparation
α-mannosidase	1	10.8	10.0 (8.96)	64 (8.52)	95.1
β-glucosidase	1020	34.7	2.3 (2.05)	2 (0.26)	12.7
α-galactosidase	400	33.8	36.1 (32.23)	240 (31.78)	98.6
β-galactosidase	30	6.1	0.5 (0.45)	1 (0.13)	28.8
β-N-acetylglucosaminidase	25	10.4	10.7 (9.55)	62 (8.31)	86.0
peroxidase	10	450	10 (10)	10 (10)	−
G6PDH	n.d.	n.d.	148 (132)	50 (6.60)	5.0
protein	−	−	−	−	14.8

oxidase from the flower tube. In *Petunia hybrida* flowers Schram *et al.* [9] demonstrated that β-glucosidase is predominantly located in the cell wall.

Using anthocyanin as vacuolar marker the proportion of hydrolase activity that is vacuolar was calculated. Table II shows that it was estimated in this way that > 95% of α-mannosidase and α-galactosidase activity is vacuolar in R27 protoplasts. Similar values for vacuolar localization of these hydrolase have been reported [2, 10].

The pH-profile of α-mannosidase and α-galactosidase activity in protoplast and vacuolar fractions was determined. It can be seen from Fig. 1 that both hydrolases show maximum activity under acidic conditions: the optimal pH of the assay being 4.0 for α-mannosidase and 5.5 for α-galactosidase activity. The pH-profiles in isolated vacuoles are identical to the profiles in protoplast fractions, which is in accordance with an exclusive vacuolar localization of these hydrolases. Based on these findings we consider α-mannosidase and α-galactosidase suited to use as vacuolar marker during the isolation and purification of vacuoles from R27 protoplasts.

In yeast cells α-mannosidase is considered a tonoplast-marker [11]. Contrastly, in tobacco cell suspensions and in *Hippeastrum* petals no association of α-mannosidase with tonoplast was detected [2, 12]. We investigated whether α-mannosidase and α-galactosidase are tonoplast-bound.

Isolated vacuoles were sonically lysed and the lysate was divided in three equal fractions. To fractions 1 and 2 Triton X-100 was added to a final concentration of 0.1%. The fractions 2 and 3 were centrifugated 30 min at 100 000 × *g* in a Beckman Airfuge. The supernate of fraction 3 was also brought to 0.1% Triton X-100.

The α-galactosidase and α-mannosidase activities were measured in fraction 1 and the supernates of fractions 2 and 3, all containing 0.1% Triton X-100. The hydrolase activities in both supernates were identical, indicating that Triton X-100 does not solubilize α-galactosidase or α-mannnosidase from pelletable membranes. Compared with the hydrolase activities in fraction 1 the supernates contained approximately 90% of both hydrolase activities. However, the pelleted 10% hydrolase activities should not be ascribed to membrane-bound enzymes, because 10–20% of the contaminating G6PDH activity (a soluble enzyme), was also pelleted.

From this experiment we conclude that neither α-galactosidase nor α-mannosidase are firmly associated with the tonoplast in *Petunia hybrida* R27 protoplasts.

Hydrolase activities were also measured in protoplast and vacuolar preparations from the mutant W78, which is derived from the line R27. The results presented in Table III show that the specific activities of the hydrolases in W78 protoplasts resemble the values found in R27 protoplasts, Table II, and that the ratio G6PDH/α-galactosidase activity is almost identical in W78 and R27 protoplasts, 4.17 and 4.09, respectively. Consequently, the

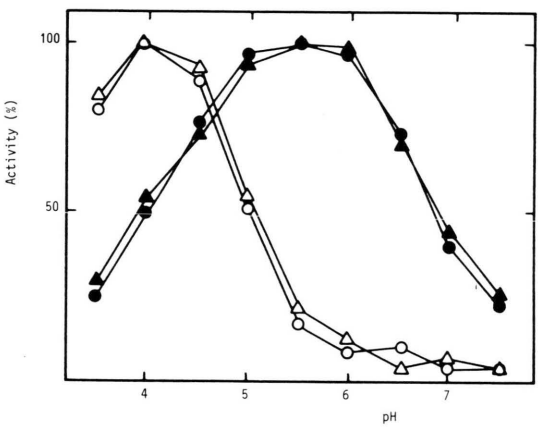


Fig. 1. pH-profiles of α-mannosidase and α-galactosidase activities in protoplast and vacuolar preparations from R27. For the range pH 3.5–6.0 0.5 M acetate and for the range pH 6.5–7.5 0.5 M phosphate buffer was used. ● = α-Galactosidase in protoplast fraction; ▲ = α-galactosidase in vacuolar fraction; ○ = α-mannosidase in protoplast fraction; △ = α-mannosidase in vacuolar fraction. 100% is the activity at optimum pH.

Table III. Specific enzyme activities of some hydrolases in protoplast and vacuolar preparations from the mutant W78, expressed in nmol/mg protein · min, and relative enzyme activities based on anthocyanin as vacuolar marker.

Activity	Protoplast preparation	Vacuolar preparation	% in vacuole [a]
α-mannosidase	9.4	18.96	97.0
β-glucosidase	33.2	1.68	1.2
α-galactosidase	30.2	129.05	100.0
β-galactosidase	0.6	0.57	22.1
β-N-acetylglucosaminidase	8.8	34.59	92.0
G6PDH	126	51	9.5
protein	–	–	23.4

[a] α-Galactosidase assumed to be 100% vacuolar.

W78 and R27 protoplasts prove to be similar with regard to hydrolases, as was expected on account of their common origin.

Assuming that α-galactosidase is 100% vacuolar in W78 protoplasts as in R27 protoplasts the vacuolar localization of the other hydrolases can be calculated. The values for vacuolar localization of hydrolases in W78 protoplasts based on α-galactosidase (α-mannosidase) as vacuolar marker (Table III) resemble closely the values found in R27 protoplasts based on anthocyanin as vacuolar marker (Table II). In our opinion these similar findings strengthen the assumption that α-galactosidase (and α-mannosidase) can be used as vacuolar marker for W78 protoplasts just as well as for R27 protoplasts.

Discussion

The polybase procedure developed for the isolation of vacuoles from upper epidermis of *Petunia hybrida* line R27 was applied to other mutant lines. Based on microscopical examination and on negative marker enzyme activity comparable vacuolar preparations could be obtained from the lines W78 and W22. However, it should be stressed that these finding do not implicate that the polybase procedure generally results in better vacuolar preparations than other methods. Isolations of qualitative good vacuoles through other procedures have been reported as well as unsuccessful attempts to isolate pure vacuoles through polybase-induced lysis [2, 10, 13]. Own observations indicate that protoplasts from petal upper epidermis of different lines of *Petunia hybrida* are differently suitable for release of vacuoles through polybase treatment. These findings illustrate that the choice of an isolation procedure strongly depend on the characteristic features of the tissue, especially the properties of the cell wall, plasmalemma and tonoplast.

The choice of a particular isolation procedure also depends on the aim of the isolation of vacuoles. Since we want to study the uptake of anthocyanin in isolated vacuoles it is especially important that the naked vacuoles do not leak anthocyanin and are capable of maintaining a proton-gradient across the tonoplast for some time. Vacuoles obtained through the polybase procedure fullfill these requirements.

In isolated vacuoles from R27 protoplasts using anthocyanin as marker the exclusive vacuolar localization of the hydrolases α-mannosidase and α-galactosidase could be demonstrated. No evidence was found for a firm association of these hydrolases with the tonoplast. The vacuolar localization of α-mannosidase and α-galactosidase can be used during the optimalization of the isolation of vacuoles from mutants which are blocked at different steps of the anthocyanin biosynthesis. Intact vacuoles that lack anthocyanin, isolated from such mutants, promise to be an attractive starting object for studying the uptake and accumulation of anthocyanin *in vitro*.

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