# Isolation of Vacuoles from the Upper Epidermis of *Petunia Hybrida* Petals

## I. A Comparison of Isolation Procedures

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Vacuoles were isolated from the upper epidermis of petals of *Petunia hybrida* line R27 using three different procedures. Utilizing the vacuolar localization of anthocyanin, vacuolar preparations obtained through polybase treatment, osmotic shock and mechanical forces were compared on purity, yield and stability. The comparison indicated that application of the polybase procedure results in the best vacuolar preparations. Vacuoles could be obtained which maintained their acidic pH and retained their anthocyanin content.

### Introduction

During the last decade several procedures have been developed for large scale isolation of intact vacuoles from plant protoplasts. Vacuoles have been released from protoplasts through osmotic shock [1–4], polybase-induced lysis [5, 6] and mechanical disruption of the plasmalemma [7–9].

There is an extensive literature concerning the isolation of vacuoles from different tissues. However, this literature shows that there exists no general agreement about the quality of vacuolar preparations obtained through different procedures. Severe criticism has been passed on vacuole isolation methods. Vacuoles obtained through osmotic shock should suffer from leakage of metabolites by stretching of the tonoplast and from resealing of the plasmalemma around the vacuole [10, 11]. It has been suggested that polybase-induced lysis is likely to result in vacuoles with a damaged tonoplast [12]. The release of vacuoles from protoplasts through very high shearing forces in some cases rather results in secondary vesicles than in the liberation of the central vacuole (personal observations). Although a considerable number of papers concerning vacuoles have been published during recent years [1–17], reports are lacking which compare different vacuole isolation methods for one type of tissue. Therefore a direct comparison can not be made up to now.

Vacuoles of upper epidermic cells of *Petunia hybrida* petals contain large amounts of flavonoids, including anthocyanins. We aim to study the function of the vacuole in the biosynthesis and metabolic channeling of anthocyanins. Wagner and Siegelman [1] briefly described the isolation of vacuoles from *Petunia* petals through osmotic shock. The procedure was worked out by Jonsson *et al.* [14] at our laboratory to study the subcellular localization of anthocyanin methyltransferase. The results from this study indicate that the terminal steps of the anthocyanin biosynthesis take place outside the vacuole, whereas these secondary plant products accumulate inside the vacuole. Similar observations were made by Hrazdina *et al.* [15, 16] in other plants studying the subcellular localization of enzymes involved in anthocyanin biosynthesis. These findings urge to study the uptake of anthocyanin in isolated vacuoles *in vitro*. Different vacuole isolation procedures were tested to determine in which manner vacuolar preparations suitable for uptake experiments can be obtained from the upper epidermis of *Petunia hybrida* petals.

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*Abbreviations:* DEAE, Diethylaminoethyl; MES, (2-[N-morpholino]ethane-sulphonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid; FDA, fluorescine diacetate; PEG, polyethylene glycol; BSA, bovine serum albumin; EGTA, ethyleneglycol-bis-(β-amino-ethylether); DTT, dithiothreitol; G6PDH, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; EDTA, ethylen diamine tetraacetic acid; NADH, nicotinamide-adeninedinucleotide.

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## Materials and Methods

### *Plant material*

*Petunia hybrida* lines 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); Dextransulphate, Mes, Hepes, FDA, PEG6000, BSA and D-mannitol from Sigma (St. Louis, USA), Metrizamide from Nyegaard (Oslo, Norway).

### *Staining with FDA*

Protoplast and vacuolar preparations were stained with FDA according to Admon and Jacoby [11], and examined by fluorescence microscopy.

### *Assays*

Anthocyanin was extracted and measured as described by Jonsson *et al.* [14]. Protein was determined according to Bradford [18]. Glucose-6-phosphate dehydrogenase and malate-dehydrogenase were assayed spectrophotometrically by monitoring changes in optical density at 340 nm [14, 19]. Fumarase and catalase were assayed by recording changes in optical density at 240 nm [20, 21]. NADH cytochrome-*c*-reductase was assayed according to Hodges and Leonard [22].

### *Protoplast preparation*

Fully developed petals of *Petunia hybrida* R27 were used for the preparation of protoplasts. Using a pair of tweezers the strongly red coloured upper epidermis was easily peeled off. The upper epidermis was vacuum-infiltrated during five minutes in incubation-medium, which consisted of 2% (w/v) cellulase and 0.2% (w/v) macerozyme in 0.6 M mannitol adjusted to pH 5.5 with NaOH. The infiltrated tissue was incubated in a Petri dish at 30 °C under constant shaking (40 strokes/minute). After three to four hours the cell walls were completely digested. The incubate was filtrated through a 50 µm nylon gauze and washed twice in 0.6 M mannitol by centrifugation at $200 \times g$ during 5 min in order to remove cell debris. The crude protoplast fraction was subsequently purified by density centrifugation in a discontinuous Ficoll gradient: the protoplasts were suspended in 20% Ficoll in 0.6 M mannitol, overlayered with 10%, 8%, 6% and 0% Ficoll in 0.6 M mannitol and centrifuged during 20 min at $1600 \times g$. Purified protoplasts were collected using a Pasteur pipette from the 0–6% Ficoll interface and washed with 0.6 M mannitol buffered at pH 6.5 with 25 mM Hepes-Tris.

### *Vacuole isolations*

Only freshly prepared protoplasts were used for the isolation of vacuoles.

#### *A. Mechanical lysis procedure*

Protoplasts were suspended in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5, containing 5% (w/v) PEG 6000, at a final concentration of approximately $10^6$ cells/ml. The suspension was sucked up eight to ten times in a 5 ml-syringe (diameter needle 25 µm), following the lysis microscopically. The lysate was mixed with an equal volume of 20% Ficoll in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5, containing 1 mM DTT and 1.6% (w/v) BSA. The suspension was overlayered with 5% and 0% Ficoll in 0.6 mM mannitol-25 mM Hepes-Tris, pH 6.5, containing 1 mM DTT and 0.8% BSA. After centrifugation during 20 min at $1600 \times g$ the 0%–5% interface was harvested using a Pasteur pipette and washed twice in the same buffer without Ficoll by centrifugation at $200 \times g$ during 5 min.

#### *B. Osmotic shock procedure*

To a concentrated protoplast suspension three volumes of lysisbuffer, consisting of 0.2 M $K_2HPO_4$, 1 mM DTT, 0.8% BSA, adjusted to pH 8.0 with HCl, were slowly added during 5 min under gentle agitation. The lysate was filtrated through a 50 µm nylon gauze and mixed with an equal volume of 20% Ficoll in 0.6 M mannitol-25 mM Tris-HCl, pH 8.0, containing 1 mM DTT and 0.8% BSA. The suspension was overlayered with 3% and 0% Ficoll in 0.45 M mannitol, 1 mM DTT, 0.8% BSA, 0.1 M $K_2HPO_4$, pH 8.0. After centrifugation during 20 min

at $1600 \times g$ the 0%−3% interface was harvested using a Pasteur pipette. The fraction was brought up to more than 10% Ficoll by adding an excess of 20% Ficoll in 0.6 M mannitol-25 mM Tris-HCl, pH 8.0, containing 1 mM DTT and 0.8% BSA. The suspension was centrifuged during 10 min at $1600 \times g$. The floating vacuoles were harvested and washed with an excess of 0.6 M mannitol-25 mM Tris-HCl, pH 8.0 containing 1 mM DTT and 0.8% BSA.

*C. Polybase-induced lysis procedure*

Protoplasts were suspended in 3 ml 20% Ficoll in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5. The suspension was overlayered with 15% Ficoll in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.0 containing 7 mg/ml DEAE-dextran and with 2 ml's of 10% and 6% Ficoll in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5 containing 3 mg/ml dextran-$SO_4$ and finally with 2 ml 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5. The tube was centrifugated during 45 min at $1600 \times g$ and the vacuoles floating at the 0%−6% interface were harvested using a Pasteur pipette. The suspension was thoroughly mixed and brought to more than 10% Ficoll by adding 20% Ficoll in 0.6 M mannitol-25 mM Hepes-Tris, pH 6.5. The suspension was overlayered with 8%, 6% and 0% Ficoll in the same buffer and the centrifugation was repeated. Purified vacuoles were collected from the 0−6% Ficoll interface.

## Results

*Isolation procedures*

From the upper epidermis of *Petunia hybrida* R27 petals large amounts of pure protoplasts can be isolated within four hours through the procedure described in Materials and Methods. Preferentially fully opened flowers of young plants were used for the preparation of protoplasts. The flower development in old plants is much slower as compared to young plants. The cell walls of upper epidermis of petals from older plants are hardly digested in the hydrolytic enzyme mixture. Much longer incubations are required for the liberation of protoplasts when petals of older plants are used. The quality of the liberated protoplasts decreases with the length of the required incubation in hydrolytic enzyme mixture. It appeared that extra-fertilization of older plants results in a faster flower development and subsequently in petals more suitable for protoplast preparation.

Because protoplasts were normally not limiting it was tried to isolate vacuoles with the lowest degree of contamination through different methods: mechanically, through osmotic shock and through polybase-treatment.

Various mechanical methods [7−9], previously described to be successful for the release of intact vacuoles from protoplasts, were tested. Using a Dounce homogenizer, filtration through glass wool or the shearing forces of centrifugation (either in the absence or presence of slightly hypotonic basic media with chelating agents as EDTA or EGTA), was not satisfactory with regard to purity of final vacuolar preparations. Only minor amounts of vacuoles were released; the ratio vacuole/protoplast always being less than 2 as examined microscopically. The purest vacuolar preparations were obtained mechanically through the excerted pressure and shearing forces of a syringe with a narrow needle.

As reported by Jonsson *et al.* [14] it is possible to release vacuoles from protoplasts from *Petunia hybrida* R27 petals through an osmotic shock in hypotonic basic media. It was observed that slowly lowering the osmolarity of a protoplast suspension was preferable to a sudden reduction by adding an excess of potassiumphosphate-buffer. The yield on intact vacuoles was higher whilst the purity of isolated vacuoles was comparable. The disruption of the plasmalemma and subsequent release of vacuoles was more gradual which improved the reproducibility of the osmotic shock procedure.

Disruption of the plasmalemma through the action of the polybase DEAE-dextran as originally described by Dürr [17] and modified for plant protoplasts by Boudet *et al.* [6] was applicable to *Petunia* protoplasts. Floatation in a polybase-lysis gradient resulted in purer vacuolar fractions as compared to centrifugation downwards the gradient as described by Boudet.

Contamination of vacuolar fractions with protoplasts is often a severe problem during the isolation of vacuoles. Due to the similarities in density and size between vacuoles and protoplasts a complete separation can often not be achieved. Such problems also occur during the isolation of vacuoles from *Petunia hybrida* petals. Attempts to get rid of contaminating protoplasts through density centrifugation in Ficoll, Percoll or Metrizamide gradients

or using aqueous two phase systems were largely unsuccessful. However, Ficoll gradients proved to be suited for the purification of vacuoles from other released cell constituents. Hence the purity of the final vacuolar preparations was mainly determined by the ratio vacuole/protoplasts after the lysis step. The transition upper epidermis cell – protoplast – vacuole during the isolation is visualized by Figure 1. Micrograph a, shows an epidermis cell liberated from the tissue after 15 min through the combined action of cellulase-macerozyme and shaking. The original pear shape of the cell can still be seen by the remaining cell wall. Probably the cell contains just one vacuole composed of several lobes surrounded by cytoplasm. Preliminary electron microscopical research presents no indications for the existence of more than one vacuole in one epidermis cell. Micrograph b, shows a naked protoplast obtained after three hours incubation in the cell wall digesting enzyme mixture. The protoplast is spherical and contains one central globular vacuole enclosed by a rind and cap of cytoplasm. In the cap the nucelus can be seen. Micrographs c and d, show typical vacuoles obtained through polybase-treatment and mechanically respectively. The average diameter of such vacuoles is identical to that of the protoplasts they are released from. Micrograph e, shows a vacuole obtained through osmotic shock. The depictured vacuole is suspended in the hypotonic lysis medium (0.15 M mannitol). The average diameter of the vacuole in such conditions is approximately 1.3 times larger than the original diameter of the vacuole in the protoplast. The surface area of the vacuole is increased approximately 100%. Whether such an enormous increase in surface area is solely accomplished by stretching of the tonoplast is uncertain. It can be deduced from a comparison of micrographs a and b, that during the protoplast preparation the surface area of the vacuole decreases. Generally, suspended protoplasts and especially vacuoles rapidly adapt their diameter when the osmolarity of their medium is changed. To explain similar observations with protoplasts of rye Wolfe and Steponkus [23] have proposed the existence of a reservoir of membrane area, able to absorb or provide membrane material during a contraction or expansion of the plasmalemma. It is tempting to suggest a similar phenomenon for the tonoplast to explain our observations.

Two other microscopical observations should be mentioned. When macerated epidermis cells (as depictured in micrograph a) are mechanically disrupted the release of two sizes of vacuoles can be observed. Usually one cell gives rise to one large and one to three small vacuoles. Probably the smaller lobes are separated from the central lobe through mechanical forces. In such a case the tonoplast should close very fast because the released vacuoles have retained their anthocyanin content.

When vacuoles are suspended they are almost perfect spherical. However, it was observed repeatedly that vacuoles which firmly sticked to the slide could be stretched four to five times their original spherical diameter without losing anthocyanin. Similar observations were made by Wagner *et al.* [24] with *Tulip* petal vacuoles.

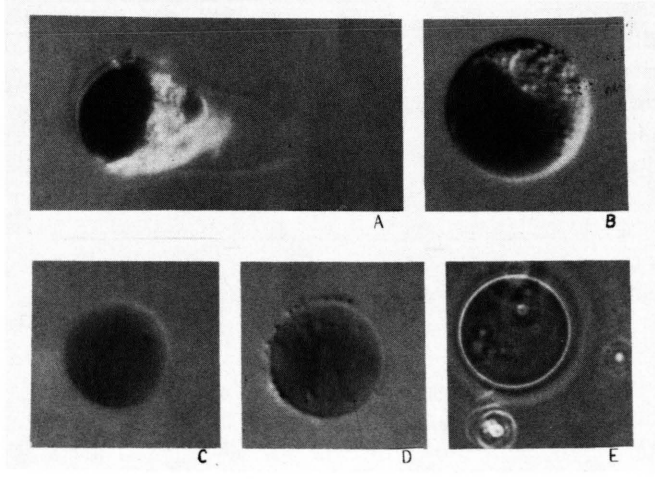


Fig. 1. Micrographs of A: liberated cell after 15 min incubation in hydrolytic enzyme mixture (1000 ×). B: liberated protoplast after 2 h incubation in hydrolytic enzyme mixture (1000 ×). C: isolated vacuole through polybase procedure (1000 ×). D: isolated vacuole through mechanical lysis procedure (1000 ×). E: released vacuole in lysis medium of osmotic shock procedure (1800 ×). a, b, c, d, suspended in 0.6 M mannitol; e suspended in 0.15 M mannitol, 0.15 M $K_2HPO_4$ pH 8.

From the microscopical observations mentioned above it can be concluded that the tonoplast of *Petunia* petal vacuoles is a very flexible membrane which can endure large deformations without losing its capacity to retain anthocyanin.

*Comparison of vacuolar preparations*

It is generally accepted that in flowers anthocyanin is exclusively located in the vacuolar compartment of the cells and consequently anthocyanin can be used as an internal vacuolar marker [25, 26]. In upperepidermis of *Petunia* petals anthocyanin can also be considered a vacuolar marker. Final vacuolar preparations obtained through three different isolation procedures were compared on purity, yield and stability of the isolated vacuoles based on anthocyanin as vacuolar marker.

*Purity*

The contamination of vacuolar preparations was determined biochemically by measuring negative marker-enzyme activities in vacuolar and protoplast fractions containing an equal amount of anthocyanin. Measured were NADH malate dehydrogenase (cytosol, mitochondria), glucose-6-phosphate dehydrogenase (cytosol), NADH cytochrome *c* reductase (endoplasmic reticulum), fumarase (mitochondria) and catalase (microbodies) activities. The data presented in Table I show that the polybase procedure resulted in vacuolar preparations with the lowest degree of contamination. The contamination with negative marker enzymes of such preparations is in the same order as reported previously for vacuoles from *Acer* and *Melilotus* protoplasts isolated through a similar procedure [6, 27]. In vacuolar preparations obtained through osmotic shock or mechanically the contamination varied between 10 and 28%. Especially the contamination with cytosol markers G6PDH and MDH was higher in such preparations as compared with vacuolar fractions obtained through the polybase procedure. The purity of vacuolar preparations was also examined microscopical with phase contrast and Normarski Interference optics. Protoplasts can be very well discriminated from vacuoles with Normanski Interference optics (see Fig. 1 b, c, d, e). In all vacuolar preparations protoplasts could be observed. The microscopically observed contamination with protoplasts was generally in good agreement with biochemical measurements. *I.e.* in vacuolar preparations showing 10% contamination based on negative marker enzyme activities the ratio vacuole/protoplast was approximately 9. This indicates that protoplasts are the main source of contamination in the final vacuolar preparations. This was confirmed using a staining method developed by Jacoby and Admon [11] to discriminate naked vacuoles from impure vacuoles which are enclosed by a plasmalemma and a small rind of cytosol, so called vacuoplasts. In final vacuolar preparations no vacuoplasts were detected. Only contaminating protoplasts were stained which could also be easily observed with Normarski Interference optics.

*Yield*

The yield on intact vacuoles was determined by measuring the anthocyanin content of the final vacuolar preparations and of the starting amount of protoplasts. The mechanical procedure resulted in very low yields. In isolations resulting in $< 20\%$ contamination with vacuolar marker enzymes, the yield was always below 1%. Using the osmotic shock procedure it was possible to isolate larger amounts of vacuoles. The yield was normally $1-5\%$. The polybase procedure resulted in the highest yield. Repeatedly $20-30\%$ of the vacuoles were recovered in the final preparations with a contamination of $5-10\%$.

*Stability*

The stability of the vacuolar preparations was determined by measuring the amount of anthocyanin retained in vacuoles during time. Freshly prepared vacuoles were placed at 4 °C in the dark

Table I. Enzyme activities related to anthocyanin in vacuolar preparations as a percentage of those in initial protoplast preparations.

| Procedure | Mechanical lysis | Osmotic shock | Polybase lysis |
|---|---|---|---|
| NADH cyt. *c* red. | 15.2 | 12.0 | 8.0 |
| G6PDH | 28.0 | 6.0 | 3.2 |
| MDH | 22.1 | 7.5 | 4.0 |
| Fumarase | 15.9 | 6.3 | 4.3 |
| Catalase | 12.1 | 4.5 | 1.9 |

Table II. Percentage anthocyanin retained in particles during time. Incubation in 0.6 M mannitol at 4 °C in the dark.

| Time [h] | Vacuolar fraction obtained through: | | |
|---|---|---|---|
| | Mechanical lysis | Osmotic shock | Polybase lysis |
| 0 | 100 | 100 | 100 |
| 1 | 102 | 96 | 98 |
| 2 | 97 | 87 | 99 |
| 3 | 97 | 83 | 95 |
| 4 | 90 | 78 | 92 |
| 18 | 68 | 18 | 78 |
| 36 | 27 | 5 | 43 |

and the anthocyanin content of pelletable particles was determined at several time intervals.

Table II shows that preparations obtained through the polybase procedure did not loose anthocyanin significantly during several hours, whereas vacuoles released through osmotic shock retained their anthocyanin to a lesser extent.

*Spectral analysis*

Absorbance scans were run for *Petunia hybrida* R27 protoplast suspensions. The contribution of light scattering and absorbance by non-vacuolar components was eliminated using a *Petunia hybrida* W78 protoplast suspension with an equal cell density as reference. The mutant W78 is derived from the cultivar R27 by a single mutation affecting the terminal steps of the anthocyanin biosynthesis, resulting in white flowers. The anthocyanin peak was found at 517 nm using buffer solution as reference and at 540 nm using a W78 protoplast suspension as reference.

Absorbance scans were also run for freshly prepared vacuolar fractions. The spectra of preparations obtained through osmotic shock and polybase-induced lysis show different optima. The anthocyanin peak is located at 580 nm and 538 nm in vacuolar fractions obtained through osmotic shock and polybase-treatment, respectively.

In order to determine the absorbance maximum of anthocyanin at different pH-values the anthocyanin was extracted from the upper epidermis of R27 petals. Spectra of extracted anthocyanin were run in 0.1 M Mes-NaOH or Tris-HCl buffers (containing NaCl to stabilize anthocyanin). Fig. 2 shows that the absorbance optima are dependent of the pH of the solution. From the absorbance maximum and the data presented in Fig. 2 the vacuolar pH in protoplasts can be estimated to be approximately 5.0. This value is in good agreement with direct measurement of the pH value of sonically lysed protoplasts: 5.1–5.2. In the same manner the vacuolar pH in vacuolar preparations obtained through osmotic shock and polybase-induced lysis can be estimated to be approximately 7.0–8.0 and 5.0, respectively. Neutral or basic pH values of vacuoles released through osmotic shock in basic media as found for *Petunia hybrida* R27 have been reported for other objects and are believed to be an artificial result of the isolation procedure [28].

It is interesting to note that the vacuolar pH in protoplasts is estimated to be less than 4.0 using the spectrum of a protoplast suspension with buffer solution as reference. The very acidic vacuolar pH values in petals of *Tulipa* and *Hippeastrum* reported by Lin and Wagner [28] using a comparable spectrophotometrical method can be ascribed to the same phenomenon.

Anthocyanin was extracted from protoplast and vacuolar preparations. The spectra hereof proved to be identical to spectra of anthocyanin extracted from stripped upper epidermis. This indicates that no modification of anthocyanin by degradation occurs during the isolation procedures.

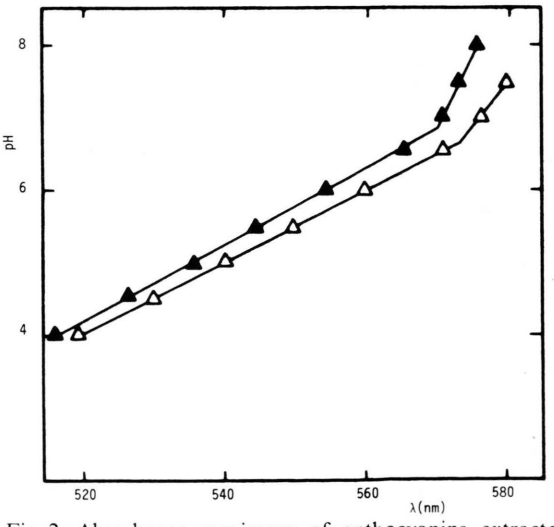


Fig. 2. Absorbance maximum of anthocyanins extracted from upper epidermis of R27 dissolved in 0.1 M Mes-NaOH or Tris-HCl. ▲: stabilized by 4 M NaCl; △: not stabilized, after 5 min in buffer.

## Discussion

The presence of anthocyanin in vacuoles of upper epidermis of *Petunia hybrida* R27 petals appeared to be convenient in several respects. Because anthocyanin can be easily extracted and accurately detected and because of its exclusive vacuolar localization it can serve as reliable vacuolar marker. On the other hand, because of the pH dependence of absorbance of anthocyanin spectral analysis of vacuoles renders information about the vacuolar pH and thereby about the quality of isolated 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.

The comparison between vacuolar preparations from upper epidermis of *Petunia hybrida* R27 petals obtained through three different procedures, indicated that the polybase procedure resulted in the best vacuoles with regard to purity, yield and stability. In contrast to vacuoles obtained through osmotic shock in basic media, the vacuoles isolated through the polybase procedure maintained their acidic pH and retained their anthocyanin for some time.

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