Isolation and Characterization of Vacuoles from Cell Suspension Cultures of *Daucus carota*

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A reliable procedure for the isolation of vacuoles from anthocyanin-containing cells of *Daucus carota* cell suspension cultures has been developed. From cells of the late linear growth phase, protoplasts were prepared and purified in a sucrose/sorbitol gradient. Vacuoles were liberated from these protoplasts by osmotic shock and purified in a Metrizamide step gradient. The vacuole-containing fractions were analysed for their anthocyanin content as a measure for the yield of vacuoles. The purity of vacuoles was examined by assaying various marker enzymes in both protoplasts and vacuoles. The purest vacuolar fraction had 8% of the total activity of glucose-6-phosphate dehydrogenase (marker for cytosol), 8% of cytochrome oxidase (mitochondria) and 10% of NADPH-cytochrome c reductase (ER). 55% of the acid phosphatase activity of the protoplasts and 35% of the total malate were recovered in the vacuoles. The vacuolar pool of amino acids is quite large. Data for 15 amino acids show that 44 to 73% are being located in the vacuole.

Though vacuoles are the largest cell organelles of plant cells, comparatively little is known about their constituents, biochemistry and function in comparison with other organelles. This is mainly due to the fragility of the vacuolar membrane, the tonoplast, that renders isolation of vacuoles very difficult. Direct analyses of the vacuolar fluid has only been performed in cases of giant cells, such as the internodal cells of the Characeae, whose sap can be obtained by micro-pipettes [1, 2]. Until recently isolated vacuoles had been available in only small numbers [3], from meristematic tissues [4, 5], or yeast [6–9].

In the last four years methods have been described for the isolation of large numbers of vacuoles from petals [10], leaves [10–12] as well as root and other storage tissue [13, 14, 28]. The vacuoles are liberated either directly by slicing the tissue [13, 28] or via enzymatically produced protoplasts which are lysed by osmotic shock [10, 12] or by polycations [11]. These methods will greatly stimulate research on the biochemistry and physiology of plant vacuoles [15].

In continuation of our studies on accumulation and degradation of secondary compounds in plant cell cultures [16], experiments on isolation of vacuoles and characterization of their constituents were started. This seemed mandatory because vacuoles are also characterized by concomitant influx and efflux of secondary compounds. This steady-state is of great importance for regulating catabolic pathways [16].

We have chosen an anthocyanin-containing cell suspension culture of *Daucus carota.* Reliable procedures for the isolation of vacuoles had to be developed by modifying known methods. In a first part of our studies the liberated vacuoles were analysed for marker enzymes, hydrolytic enzymic activity, malate and amino acids.

Abbreviations: ER, endoplasmatic reticulum; 2,4-D, 2,4-dichlorophenoxyacetic acid; BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid; DTE, dithioerythritol

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Materials and Methods

Plant Material

Suspension cultures of *Daucus carota* L. were established from an anthocyanin containing callus (strain Dcb) [17, 18]. The cells were cultivated in 40 ml medium according to Murashige and Skoog [19] (MS medium) supplemented with 0.5 mg/l 2,4-D in 200 ml Erlenmeyer flasks in continuous light (7600 lux) on a gyrotory shaker (120 rpm). 1 g inoculum was transferred to fresh medium every 7 days. Such cultures reached stationary phase around day 10 and showed a 5fold increase in fresh weight during that period.

Isolation and purification of protoplasts

5 g cells (fresh weight) taken from the linear growth phase (7 days) were incubated in 100 ml MS medium containing 2% (w/v) cellulase (Onuzuka R 10, Kinki Yakult Manuf. Co. Nishinomiya, Japan) and 0.6 M sorbitol at 25 °C, 7600 lux and 120 rpm for 15 hours. The liberated protoplasts were centrifuged at 600×*g* for 10 min, resuspended in MS medium containing 0.6 M sorbitol and were twice recentrifuged.

The crude protoplast suspension thus obtained was purified by centrifugation (600×*g*) on a discontinuous sucrose/sorbitol gradient in which the steps contained sucrose and sorbitol in MS medium in a concentration of 21 : 0, 12 : 5 and 8 : 7% (w/v). The obtained protoplast interfaces were combined and the pellet was discarded. This pure protoplast suspension was diluted with two volumes of MS medium containing 0.6 M sorbitol, and the protoplasts were concentrated by centrifugation (600×*g*, 10 min) for the further isolation of vacuoles. For assaying enzymes and constituents of protoplasts a small aliquot was separately diluted with the below mentioned isolation medium

The digestion of the cell wall during liberation of protoplasts could be observed in a fluorescence microscope after staining with Calcofluor White ST (American Cyanamid Co, Bound Brook, N. J.).

Isolation and purification of vacuoles

The vacuoles were liberated from protoplasts by osmotic shock in a buffer containing 0.15 M sorbitol. 3 mM $MgCl_2$, 0.5% (w/v) BSA and 1 mM HEPES (pH = 8.0). After incubation for 5 min the crude vacuole suspension was layered on a Metrizamide step gradient consisting of 2.0 ml fractions of 2, 4, 8 and 16% (w/v) Metrizamide (2-3-acetamido-5-N-methyl-acetamido-2,4,6-triodobenzamide-2-desoxy-D-glucose) (Molter, Heidelberg), 0.5% BSA and 0.55 M sorbitol in 1 mM HEPES (pH = 8.0). Centrifugation was carried out at 100,000×*g* for 2 hours in a swing out rotor. The deeply-coloured vacuole containing fractions were separately analyzed.

Vacuoles and protoplasts were counted using a Fuchs-Rosenthal hemocytometer.

Assays

Vacuoles and protoplasts were lysed by adding the buffers needed for the further enzymatic assays and stirring with a magnetic stirrer in test-tubes for 30 min. After centrifugation (5000×*g*) the supernatant was used for the assays.

An 0.2 ml aliquot of the vacuolar fraction was extracted with 0.8 ml methanol containing 1% HCl, and the absorbance measured in 1 cm cuvettes at 530 nm. The absorption value of the aliquot was multiplied with the total volume of the vacuolar fraction thus yielding, in absorption units, a measure for the total anthocyanin content.

Glucose-6-phosphate-dehydrogenase was assayed according to Bergmeyer *et al.* [20], while cytochrome oxidase and NADPH-Cyt c reductase were measured according to Hodges and Leonard [21].

Hydrolyses were measured using nitrophenyl derivatives of various substrates. After incubating for 30 min at 30 °C the reaction was stopped by 1 M Na_2CO_3 and the absorption was measured at 400 (*p*-nitrophenolate) and 420 nm (*o*-nitro-phenolate), respectively, both against enzyme and substrate blanks. Acid phosphatase was assayed in 0.025 M acetate buffer (pH = 5.5) with *p*-nitrophenylphosphate, while α- and β-glucosidase, β-glucuronidase and β-galactosidase were recorded in 0.05 M citrate buffer (pH = 5.0) with *p*-nitrophenyl-α-D-glucoside, -β-D-glucoside, -β-D-glucuronide, and *o*-nitrophenyl-β-D-galactoside, respectively. Esterase was measured in 0.07 M phosphate buffer (pH = 7.0) with *o*-nitrophenylacetate as substrate.

Amino acids were assayed with an amino acid analyzer (Biocall BC 200). An Aminex A-6 (Bio-Rad Lab./Richmond) 17,5 v column was used. Malate was measured enzymatically according to Möllering [22].

Results

Isolation of vacuoles

The Dcb callus strain of *Daucus carota* [17, 18] when turned into rapidly growing cell suspensions still produced anthocyanin pigments in quite high yield (app. 2 μmol/g fr.w.). Anthocyanin formation occurred practically parallel to growth. The pigments are known to be deposited in the vacuoles [15] as can easily be checked microscopically in plasmolyzed cells or in liberated vacuoles.

To measure the yield of vacuoles we analysed the anthocyanin content of the various fractions obtained. This proved to be more reproducible than

Table I. Distribution of anthocyanin and activity of marker enzymes in fractions of a Metrizamide step gradient.

Fraction *	Anthocyanin	Glucose-6-phosphate dehydrogenase		Cytochrome c oxidase		NADH-cytochrome c oxidoreductase	
	[%]	[mU/AU]	[%] °	[mU/AU]	[%] °	[mU/AU]	[%] °
0/2	31	48.7 ± 12.8		0.3 ± 0.3		77.0 ± 20.4	
2/4	19	3.0 ± 1.8	14	0.5 ± 0.5	12	6.6 ± 3.1	12
4/8	21	1.5 ± 1.6	8	1.2 ± 0.4	10	5.5 ± 2.2	10
8/16	22	1.3 ± 1.6	7	7.6 ± 5.7	11	6.3 ± 2.0	11
Pellet	7	0.3 ± 0.3		158 ± 32		246 ± 58	
Protoplasts	100	18.8 ± 5.8	100	17.5 ± 3.0	100	55.4 ± 8.4	100
N	5	5		3		3	

AU, absorbance unit of anthocyanin at 530 nm (1 AU in 1 ml of the original sample gives an extinction of 1.000).
N, number of separate experiments in which marker enzymes were assayed.
°, compared to protoplasts (= 100%).
*, figures indicate Metrizamide concentration.

counting the number of vacuoles. We based all data for enzyme activity and vacuolar content on the degree of pigmentation. This is especially mandatory since vacuoles of all sizes up to 60 μm in diameter were obtained. The variation in size of vacuoles is partly due to the fact that in a non-synchronized suspension culture large mature cells with one central vacuole cooccur with smaller cells still containing several vacuoles. Furthermore, during the process of purification the number of vacuoles tends to increase by budding phenomena of larger vacuoles. Thus the anthocyanin content of the *D. carota* cells is a suitable internal standard.

For the isolation of vacuoles we first tried 0.2 M Na_2HPO_4 with 3 mM $MgCl_2$ and DTE as hypertonic solution [10]. Better results were obtained with 0.15 M sorbitol, 3 mM $MgCl_2$, 1 mM HEPES and 0.5% (w/v) BSA. With DTE we observed pasting of cytoplasm and vacuoles, that renders purification very difficult. For gradients we tried sucrose, mannitol, Ficoll and Metrizamide. The latter gave the best results.

Vacuoles were obtained by osmotic shock of protoplasts and purified on a Metrizamide multi-step gradient. Vacuoles were collected in three separate bands at the interfaces of the step gradient representing three density ranges. Table I shows the distribution of anthocyanin and the activity of several marker enzymes in the separated fractions of this Metrizamide gradient.

The anthocyanin content was estimated to be essentially proportional to the yield of vacuoles. However, the upper fraction (0.2%) contains more free anthocyanin which mainly stems from vacuoles which have burst during liberation. Marker enzyme activities of the different fractions were based on the absorbance unit of anthocyanin and can thus be compared with the values obtained for protoplasts. One absorbance unit corresponds to 190,000–210,000 protoplasts and ca. 10^6 vacuoles of the fractions 2/4, 4/8, and 4/16 (vacuolar bands of the Metrizamide gradient). As explained above these figures indicate that more than one vacuole per protoplast was isolated. Clear differences between the three vacuolar bands with regard to size or anthocyanin content could not be observed. Protein and/or ion content of a vacuole appears to be an important factor determining the density of the vacuoles. The three vacuolar bands are therefore presumably due to the cellular variation in a suspension culture.

To examine the degree of contamination of the vacuoles with protein material from other cell organelles, activities of three marker enzymes were assayed, namely glucose-6-phosphate dehydrogenase for cytosol [13], cytochrome oxidase for mitochondria [13, 21] and NADPH-cytochrome c reductase for endoplasmatic reticulum [21].

As based on enzyme activity/unit anthocyanin glucose-6-phosphate dehydrogenase mainly remains in the upper fraction. The interesting 3 vacuolar bands still contain 7 to 15% of the activity assayed in the protoplasts. Cytochrome oxidase is mainly found in the pellet, though the vacuolar band 8/16 is still slightly contaminated. NADPH-cytochrome c reductase has its main activity both in the upper fraction and in the pellet. The activity of this enzyme in the vacuolar bands still amounts to 10 to 12% of that of the protoplasts. Thus, the purest fraction 4/8, in

which 21% of the anthocyanin of the original protoplasts were recovered, shows 8% residual activity of the glucose-6-phosphate dehydrogenase and 10% each of the cytochrome oxidase and the cytochrome c reductase of the protoplasts. Therefore, a slight contamination of the vacuoles has to be considered for the further investigations. The sum of the absolute activities of the enzymes in the gradient fractions amounted to 70 to 90% of the activities measured with the protoplasts.

Constituents of vacuoles

Vacuoles of the purest fraction 4/8 were analysed for their hydrolase activity, and their content of amino acids and malate.

Table II. Activity of hydrolases in protoplasts and vacuoles from *Daucus carota* cell suspensions.

Enzyme	Activity [mU/AU]		
	Protoplasts	Vacuoles (4/8)	
		measured	corrected *
α-Glucosidase	<0.5	<0.5	–
β-Glucosidase	<0.5	<0.5	–
β-Glucuronidase	<0.5	<0.5	–
β-Galactosidase	1.7	0.7	0.6
Esterase	0.5	<0.5	–
Acid phosphatase	28.3	18.5	16.7

* Because of the enzymatically estimated degree of contamination (see Table I) the measured data were corrected by 10%.

Table III. Content of soluble amino acids in protoplasts and vacuoles as found in fraction 4/8 (see Table I).

Amino acid	Protoplasts	Vacuoles		
		measured	corrected *	
	nmol/AU	nmol/AU	nmol/AU	%
Alanine	52.5	34.4	29.1	55
Glutamic acid	38.8	22.2	18.3	47
Proline	19	10	8	40
Valine	18.8	10.1	8.2	44
Leucine	9.6	6.4	5.4	56
Isoleucine	7.9	5.5	4.7	59
Aspartic acid	7.7	5.9	5.1	66
Histidine	7.1	5.9	5.2	73
Glycine	6.0	4.7	4.1	68
Lysine	5.6	3.4	2.8	50
Arginine	4.0	2.6	2.2	55
Tyrosine	3.6	2.0	1.6	44
Methionine	1.2	<0.5	–	–
Phenylalanine	0.8	<0.5	–	–
Cysteine	<0.5	<0.5	–	–

* See footnote in Table II.

Table IV. Content of malate in protoplasts and vacuoles from *D. carota* cell suspensions.

Amino acid	Protoplasts	Vacuoles		
		measured	corrected *	
	nmol/AU	nmol/AU	nmol/AU	%
Malate	119	54	42	35

* See footnote in Table III.

Table II shows the measured hydrolase activities for both protoplasts and vacuoles. α- and β-glucosidase, glucuronidase and esterase are close or under the detection level. With α-galactosidase about 30% of the activity found in the protoplasts was regained in the vacuolar fraction. The absolute values are, however, low and must be interpreted with caution. More significant data were found for acid phosphatase. Here, about 55% of the activity measured in protoplasts is located in the vacuoles. Vacuoles must be expected to be a major site for amino acid accumulation which is rather well documented for yeast [9, 15]. Protoplasts and vacuoles from *Daucus carota* cell suspension cultures were analysed for their free amino acids. Table III shows these data for protoplasts and vacuoles. The amino acids are listed according to their quantity in the protoplasts and their amount in vacuoles is again based on anthocyanin content. According to the corrected values 44 to 73% of the amino acids found in the protoplasts are located in the vacuole. This requires for most of the amino acids that the bigger part is separated from direct cytoplasmatic metabolism. The amino acids with the largest relative vacuolar pool is histidine, followed by glycine and aspartic acid. The smallest relative pool was found for proline, valine, and tyrosine. The absolute amounts of methionine, phenylalanine and cysteine, respectively in either protoplasts or vacuoles is very low. A distribution of amino acids correlated with some specific properties, for example basicity of amino acids, is not evident from Table III. As an example for organic acids the amount of malate in both protoplasts and vacuoles was determined. In this case, some 35% of the content found in the protoplasts was isolated from the vacuoles (Table IV).

Discussion

After various attempts a reliable procedure for vacuole isolation could be established. Using osmotic

lysis of protoplasts and subsequent centrifugation in a Metrizamide gradient three fractions of vacuoles were obtained which contain 19, 21, and 22% of the anthocyanin present in the protoplasts. The assumption that the anthocyanin in the gradient is exclusively located in vacuoles seems experimentally justified. Therefore, based on the anthocyanin data about 60% of the vacuoles originally present in the protoplasts were finally found in the vacuolar fractions. With respect to purity it was shown that absolutely clean vacuoles could not be obtained (Table I). One reason may be an inhomogenous cell material in the suspension cultures. There may also be some unbroken protoplasts possessing the same density as the vacuoles which contribute to this contamination. Other published data on marker enzymes of vacuoles isolated from different cell material also showed slight enzymic contamination [4, 13]. In our case of anthocyanin containing *D. carota* cells we possess a simple basis to compare protoplasts and vacuoles. As a measure for comparison and for contamination of the vacuoles it is recommended to assay the marker enzymes and calculate their relative portion and the anthocyanin basis (Table I and II). All further investigations should be interpreted with regard to this degree of contamination.

In vacuoles of meristematic root cells and of yeast cells Matile [4, 5] and Matile and Wiemken [8] found a number of acid hydrolytic enzymes, which led to the assumption [15] that the vacuole is the compartment of cellular digestion, and thus similar to animal lysosomes.

This concept of proteolytic function of vacuoles has recently been substantiated for endosperm from castor bean seedlings by Nishimura and Beevers [14]. On the other hand Butcher *et al.* [23] in their studies on vacuoles prepared from *Hippeastrum* petals reported data which are not consistent with this concept of a generalized lysosomal nature of plant cell vacuoles. In the vacuolar lysate of these mature plant cells they only found acid phosphatase, RNase, and DNase. Because of the very low enzymic activity in the *Daucus carota* cells we could only estimate the distribution of acid phosphatase with any degree of certainty. Our results show that 55% of the activity measured in protoplasts was located in the vacuoles. This corresponds well with the findings by Butcher *et al.* [23] who measured 49% of this particular activity in the vacuolar lysate. Nishimura and Beevers [14] also found acid phosphate not to be confined to the vacuole though they consider it quite possible that six of the hydrolases tested are entirely localized in the vacuole. With regard to the lysosomal function of vacuoles more investigations on isolated vacuoles are needed, that particularly deal with possible alterations in the function of vacuoles during maturation of the cells. Buser and Matile [11] reported for the CAM plant *Bryophyllum daigremontianum* that more than 80% of the cellular malate was located in the vacuoles of mesophyll cells. For *Daucus carota* vacuoles we only found 35%. The difference may be due to the much higher total amount of malate in this *Bryophyllum* species. Furthermore, there is a difference in metabolic function of malate between a plant showing crassulaceen acid metabolism and an heterotrophic cell suspension culture.

Very little is known about the vacuolar pools of amino acids in higher plant cells. Almost all data were obtained with yeast vacuoles [9, 24–26]. These investigations showed that the bulk of the free amino acids is located in the vacuole. Thus, for *Candida utilis* 83% [25] and for *Saccharomyces cerevisiae* 60% [26] of the total amino acid pool was found in the vacuole. Some amino acids like arginine even reached up to 93% [25]. Using pulse-labelling experiments Wiemken and Nurse [25] were able to show that the vacuolar amino acid pool had a slow turnover, whereas the cytoplasmatic pool had a rapid turnover. For higher plants results of feeding experiments have indicated the existence of two amino acid pools [27]. There is also some evidence that the more active amino acid pool makes up less than half of the total pool. So far studies on isolated vacuoles from higher plants have in only one case shown the existence of amino acids in vacuoles. Buser and Matile [11] reported that vacuoles from *Bryophyllum daigremontianum* contain about 50% of the amino acids found in the protoplasts [11]. Our data (Table III) on *Daucus carota* cells appear to be the first detailed analyses about the vacuolar composition and vacuolar pool size of the proteinogenic amino acids in plant cells. Though the high quantities found for yeast vac uoles are not reached, the vacuolar pools of the soluble amino acids of the *Daucus carota* cells are remarkably high. Such data are important for further investigations on amino acid metabolism in plant cells. The very low value found for phenylalanine (Table III) is not surprising, because the cells were worked up a time of maximum phenylpropanoid biosynthesis.

Isolated vacuoles will in future provide a useful experimental tool for investigating vacuolar accumulation and transport processes of amino acids and secondary metabolites across the tonoplast. Especially with vacuoles from cell suspension cultures there is a wide experimental field because in these cells accumulation and degradation of secondary constituents can easily be manipulated by phytohormonal treatment [16].

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