ELSEVIER

Journal of Chromatography A 817 (1998) 337–343

JOURNAL OF CHROMATOGRAPHY A

Analysis of solutes and metabolites in single plant cell vacuoles by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) is demonstrated as a useful technique for the determination of a large number of solutes and metabolites such as inorganic anions, carboxylic acids, sugars and amino acids in single plant cell vacuoles. Sample volumes in the picoliter range can be extracted from single vacuoles and divided into subsamples for subsequent analyses. The challenges involved with such low volume samples are the low analyte amounts present, which require CE systems with sensitive detection approaches for the different compounds, and the danger of contamination and evaporation, which makes minimized sample handling under an inert protective medium essential. Three different separation and detection approaches were used in this investigation. For the determination of inorganic anions and carboxylic acids in vacuolar samples, indirect UV detection using salicylic acid as background electrolyte provides sufficient sensitivity. Amino acids were derivatised on-column with *o*-phthaldialdehyde-2-mercaptoethanol, separated by micellar electrokinetic chromatography and detected with fluorescence. For sugars a separation and direct UV detection based on chelation with Cu(II) was employed. The determination of these compounds in single vacuoles of epidermal or mesophyll cells of wheat leaves is shown.

Keywords: Plant materials; Inorganic anions; Carboxylic acids; Sugars; Amino acids

1. Introduction

The knowledge of the local distribution of solutes and metabolites between different cells and their changes due to ambient stimuli is a prerequisite for the understanding of intercellular communication and the regulation of the solute and metabolite transport in plants. In recent years evidence was supplied, that compounds not only show a strong compartmentation between vacuole and cytoplasm of a cell, but are also differentially distributed between different cell types [1] and even between cells of the same tissue [2–4]. Consequently, plant tissues cannot be considered as homogeneous material, but show distinct patchiness. One has to realize, that with the analysis of bulk tissue samples, only mean solute and metabolite concentrations of all compartments and different cell types are obtained, which do not reflect the fine scale patterns between adjacent cells or provide information about processes, that are regulated on the single cell level. Such investigations essentially require single cell or even subcellular analyses.

Several approaches have been made for this purpose, such as the direct measurement of ions in single plant cells by ionselective microelectrodes [5–7], energy dispersive X-ray (EDX) analysis of frozen tissue sections [8–10] or extracted vacuolar sap [4,11] and micro-fluorometric enzymatic assays of cell sap [4,11]. However all these methods have intrinsic drawbacks, which limit their applicability.

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0021-9673/98/$19.00
PII: S0021-9673(98)00383-5

Microelectrodes only allow the analysis of one or at best a few inorganic ions simultaneously. The information obtained by EDX are rather limited, as only elements are determined, hence the corresponding chemical species or the soluble and insoluble parts of a sample cannot be distinguished. For micro-spectrofluorometric analysis enzymatic assays for a wide variety of solutes are available. However, simultaneous determination of analytes is not possible and cross-reactivity may occur in complex samples.

In the last few years capillary electrophoresis (CE) has been introduced as a powerful technique for phytochemical analysis [12–19]. Using the high efficiency of CE the simultaneous determination of many compounds can be achieved even in complex plant matrices. The low injection volumes required (in the nanoliter-range for 75 and 50 μm capillaries and even less for capillaries with smaller I.D.) opened the possibility of analyses on the single cell level as shown by various approaches like the analysis of whole single snail neurons [20–23], human erythrocytes [24–29] or other mammalian cells [30–33] and analysis of cytoplasm extracted from individual cells [21,34–37]. Several reviews on this topic have been published [38–42].

Recently we reported a technique for sampling and handling of sap from individual vacuoles, including sample acquisition, creation of subsamples of approximately 10 picoliters, mixing with an internal standard of exactly the same volume for calculation of solute concentrations, transfer of the subsample/internal standard droplets into a CE capillary and subsequent analysis for inorganic cations and anions using indirect UV detection [43]. This method allows several analyses of subsamples from the same vacuole and thus, in principle, determination of a large number of compounds in individual vacuoles.

In this work we demonstrate the determination of inorganic anions, carboxylic acids, amino acids and sugars in vacuolar samples using capillary electrophoresis. All these compounds lack suitable chromophores or fluorophores, thus direct detection is not possible. The determination of such components in picoliter volume samples is even more challenging, as manipulation of picoliter volumes is elaborate and must be performed under paraffin oil to prevent evaporation and contamination. Therefore common sample pretreatment methods for large volume samples, e.g. extraction, filtration or enrichment techniques are not suitable with picoliter volumes. For the same reason detection methods based on precolumn derivatisation are difficult to perform. In addition, apolar derivatisation reagents or derivatisation products might be partitioned between the sample droplet and the protective paraffin oil, thus leading to analyte loss and to contamination of adjacent sample droplets.

Three different approaches are shown to be suitable for the analysis of these solutes and metabolites in vacuolar samples, based on (1) indirect UV detection, (2) direct UV detection after dynamic labeling and (3) fluorescence detection after on-column derivatisation (at the capillary inlet). None of the three methods require further sample pretreatment prior to CE. For carboxylic acids indirect detection using salicylate as background electrolyte provides sensitivity sufficient for their determination in vacuolar samples.

For amino acid determination the fast reaction with *o*-phthaldialdehyde-2-mercaptoethanol was employed for on-column derivatisation [44], followed by separation with micellar electrokinetic chromatography and fluorescence detection.

The analysis of sugars (in the vacuole mainly glucose, fructose and sucrose) was performed by dynamic labeling based on chelation of Cu(II). This method was recently described [45] and provides both, separation of the electrophoretically migrating chelates and direct UV detection due to an inner-ligand band caused by the bonds between Cu(II) and the hydroxy groups of the sugars.

2. Experimental

2.1. Apparatus

The experiments were carried out on laboratory-built CE systems, equipped with either a Lambda 1000 UV detector (Bischoff, Leonberg, Germany) or a Jasco FP-920 fluorescence detector (Jasco) and a high voltage power supply type HCN 6 M - 30 000 from FUG (Rosenheim, Germany).

Untreated fused-silica capillaries (Chromatographie Service, Langerwehe, Germany) with 50 or

75 μm I.D.×360 μm O.D. were used. The total lengths of the capillaries varied from 70 to 100 cm. On the injection side 2 cm of the polyimide coating was removed, and the outer diameter of the capillary tip was conically reduced to about 150 μm by manual grinding on a fast rotating corundum plate.

Two types of microcapillaries were used for sampling and for measuring identical aliquots of sample and internal standard. Capillaries for cell sap sampling were pulled from borosilicate glass capillaries with 1.0 mm O.D.×860 μm I.D. (Clark Electromedical Instruments, Pangbourne, Reading, UK) to a tip diameter of about 1 μm using a vertical pipette puller Model 700C (David Kopf Instruments, Tujunga, CA, USA). Constriction capillaries were prepared from the normal sampling tips with a microforge.

Droplets of volumes in the nanoliter range were produced using an ultra micro pump (World Precision Instruments, Berlin, Germany) equipped with a modified microsyringe (ILS, Stützerbach, Germany) with luer connected glass microcapillary.

Sample injection was carried out by moving the end of the separation capillary with a micromanipulator (Leitz, Wetzlar, Germany) towards the sample droplet. All operations were observed with a stereoscopic microscope (Nikon SMZ-1B, Düsseldorf, Germany).

2.2. Chemicals

For sample storage and treatment AS 4 silicone oil (Wacker, Burghausen, Germany) and paraffin oil (type 76235, Fluka, Buchs, Switzerland) were used. Both were ultrasonically extracted 20 times with Milli-Q water (18 MΩ) to remove ionic impurities and centrifuged.

The electrolyte for the determination of inorganic anions and carboxylic acids contained 7.5 m*M* salicylic acid (Fluka), 15 m*M* tris(hydroxymethyl)aminomethane (Fluka) and 1 m*M* DoTAOH. The latter was prepared from the bromide salt (Fluka) using an anion-exchanger in the hydroxide form.

Amino acids were derivatised with *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (2-ME), both purchased from Fluka.

For MEKC separation of the amino acid derivatives an electrolyte with SDS and sodium tetraborate (both from Merck, Darmstadt, Germany) was used.

The electrolyte for sugar analysis contained 7 m*M* copper(II) sulphate (Fluka) and 650 m*M* ammonia, prepared from 25% ammonia solution (Merck), pH was 11.53. All electrolytes were dissolved in Milli-Q-water and filtered through a 0.45 μm filter.

All standard substances were purchased in the highest quality available, inorganic anions (as their sodium salts), carboxylic acids and sugars from Merck and amino acids from Fluka.

2.3. Plant material

Wheat seedlings were grown under hydroponic conditions (modified Long Ashton medium with 7 m*M* sulphate and 0.9 m*M* nitrate) and 70 $\mu mol/m^2$ s light intensity. Vacuolar samples were taken from the upper epidermis or through the stomata from mesophyll cells of the third leaf of three-week-old plants.

2.4. Sample acquisition and pretreatment

The entire procedure of sample acquisition and handling of single vacuole samples is described in detail elsewhere [43]. Briefly, vacuolar sap is extracted with a silicone oil filled microcapillary. The sample droplet is transfered into a Petri dish, filled with paraffin oil. Subsamples, each approximately 10 to 20 pl, are created from the vacuolar sample using a constriction capillary. The subsamples are injected together with identical volume of internal standard (prepared with the same constriction capillary) into 5 nl water droplets, also present in the Petri dish. These water droplets, containing identical volumes of vacuolar sap and internal standard are injected into the CE capillary and analysed. All these sample handling steps are manipulated under paraffin oil, to prevent contamination and evaporation of the sample droplets.

The internal standard peak in the respective run is employed to calculate the concentrations of the different compounds using a normal calibration of analytes and internal standard with hydrostatic or hydrodynamic injections.

3. Results and discussion

The procedure of creating subsamples from a vacuolar sample described in the Experimental section allows on the one hand repeated injections into the same CE system to achieve high reliability of the data or on the other hand the use of different electrolytes and detection approaches to provide determination of a large number of compounds in individual vacuoles.

3.1. Determination of inorganic anions and carboxylic acids

In all plant samples, and also in the vacuoles, inorganic ions are by far the compounds of the highest concentrations (in the millimolar range). Carboxylic acids occur in concentrations one or even two orders of magnitude below. Nevertheless, CE is capable of simultaneous determination of both classes of compounds using indirect UV detection. Since the concentration difference between anions and carboxylic acids requires higher sensitivity for the latter, the mobility of the employed UV-active electrolyte co-ion should match the mobility of the (slower) carboxylic acids, according to the equations by Yeung [46] and Nielen [47]. This is given by salicylate, whose mobility at pH 8.1 closely matches that of propionate [18]. Reversal of the electroosmotic flow (EOF) using DoTAOH as modifier provides the same migration direction of the fast inorganic anions and the slow carboxylic acids. In Fig. 1 the determination of anions and carboxylic acids in a subsample of an epidermal vacuole of a wheat leaf is shown. The corresponding vacuolar sample had a volume of about 60 pl, measured under the microscope against the scale of an eyepiece graticule. Subsamples of about 10 to 20 pl were created and internal standardization with 200 m*M* bromide was performed using the same constriction capillary. This internal standard was used to calculate the analyte concentrations in the original vacuolar sample using a normal calibration for bromide and the analyte components with hydrostatic injections.

In addition, the internal standard (I.S.) signal can now not only be applied to calculate analyte concentrations, but also allows the exact determination

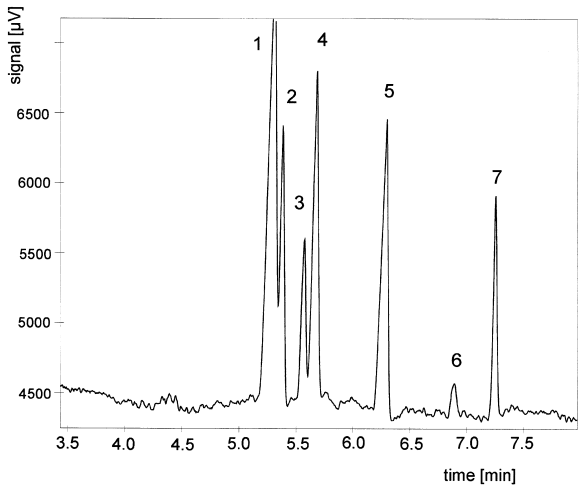

Fig. 1. Determination of inorganic anions and carboxylic acids in a subsample of an epidermal vacuole of a wheat leaf; Peak: 1=Br^-, 2=Cl^-, 3=SO_4^{2-}, 4=NO_3^-, 5=?, 6=malate, 7=phosphate; Electrolyte: 7.5 m*M* salicylic acid, 15 m*M* Tris, 1 m*M* DoTAOH, pH 8.1. Capillary: 99.5 cm (80 cm to detector)×75 μm I.D.×360 μm O.D., 150 μm O.D. at the capillary inlet. Conditions: voltage −25 kV, current 9 μA. Detection: indirect UV, 232 nm.

of the volume of the constriction capillary (from the tip to the constriction) and thus calibration of the latter. Parameters required for this calculation are the original concentration of the I.S. ($c_{\text{I.S.}}^{\text{orig}}$), in this case 200 m*M* bromide, and the hydrostatically injected volume ($V_{\text{inj, hydrost.}}$) of the calibration standards, which is calculated from the values of height difference, injection time and viscosity. With the observed value of I.S. concentration ($c_{\text{I.S.}}^{\text{obs}}$), obtained from the sample droplet, the volume of the constriction capillary ($V_{\text{constr.}}$) can be calculated according to Eq. (1).

$$V_{\text{constr.}} = \frac{c_{\text{I.S.}}^{\text{obs}}}{c_{\text{I.S.}}^{\text{orig}}} \cdot V_{\text{inj, hydrost.}} \quad (1)$$

where $V_{\text{inj, hydrost.}}$ = injection volume of the standards used for calibration; $c_{\text{I.S.}}^{\text{obs}}$ = observed value for I.S. concentration obtained from the calibration; $C_{\text{I.S.}}^{\text{orig}}$ = original concentration of internal standard.

Once calibrated this way, there is no more reason for internal standardization in order to calculate analyte concentrations, if this constriction capillary is repeatedly used. This is possible, since volume measurement with a constriction capillary can be

performed with high accuracy, allowing the repeated creation of exactly identical volumes, as shown in previous work [43].

From the bromide signal in Fig. 1 a concentration $c_{\text{I.S.}}^{\text{obs}}$ of 122 μ*M* is calculated, corresponding to an injection volume $V_{\text{inj, hydrost.}}$ of 23 nl. Thus, the volume of the constriction capillary was 14 pl. This calibrated constriction capillary was used for all subsequent analyses.

3.2. Determination of amino acids

As most amino acids occur in much lower concentrations in vacuoles compared to carboxylic acids, on-column derivatisation (at the capillary inlet) is required to form detectable products in this case. Fluorescent derivatives are preferable over UV-absorbing species due to the higher selectivity of fluorescence detection and the possibility of high excitation intensities using laser induced fluorescence. The well-known OPA-2-ME reaction [48] was used for this purpose. This reaction provides several advantages: (1) the reagents themselves show no fluorescence, thus no system peaks can interfere with fluorescence detection and excessive amounts of derivatisation reagents can be employed, (2) reaction is fast and can be performed on-column using sandwich injection, (3) separation of the isoindoles, thus formed, is possible with MEKC. In Fig. 2 the separation of amino acids in a vacuolar sample from an epidermis cell of a wheat leaf is shown. The same constriction capillary was used as for the carboxylic acids, therefore, as already mentioned, no internal standard addition was necessary. The procedure of sample injection was modified in this case, in order to achieve the on-column derivatisation. In the first step a derivatisation zone containing OPA and 2-ME is injected hydrostatically, followed by the water droplet containing the vacuolar sample. After that a second derivatisation zone is injected, thus the sample droplet is totally encapsulated by derivatisation solution. Mixing of the zones by diffusion and reaction is allowed for an appropriate time (calculated from the diffusion coefficients and the zone lengths) and then the high voltage is switched on to start the separation of the derivatised amino acids [44]. In order to achieve a fast mixing of sample and derivatisation zones, the size of the water droplet

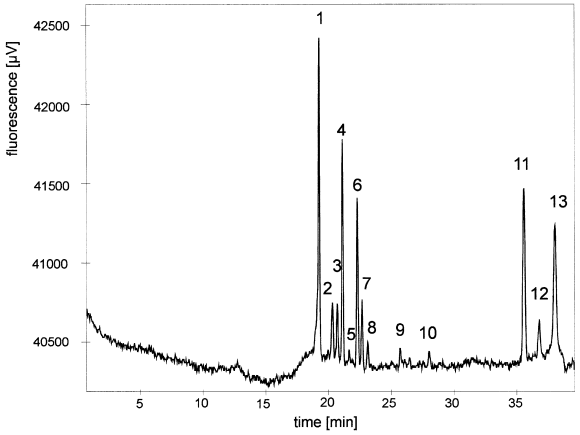

Fig. 2. Determination of amino acids in a subsample of an epidermal vacuole of a wheat leaf using MEKC and fluorescence detection after on-column derivatisation with OPA-2-ME; the same constriction capillary for creation of the subsample is used as in Fig. 1. Peak: 1=Gln, 2=Ser, 3=?, 4=Ala, 5=Gly, 6=Val, 7=?, 8=Met, 9=?, 10=Leu, 11=Glu, 12=?, 13=Asp. Derivatisation: (1) hydrostatic injection of derivatisation solution (3.7 m*M* OPA, 20 m*M* sodium tetraborate, 5 m*M* 2-ME, 10% (v/v) MeOH), 7 cm, 20 s; (2) injection of sample droplet (total volume 1 nl); (3) injection of derivatisation solution, 7 cm, 20 s; (4) 3 min reaction time. Electrolyte: 27 m*M* sodium tetraborate, 44.5 m*M* SDS, 5% (v/v) acetonitrile. Capillary: 101 cm (82 cm to detector)×50 μm I.D.×360 μm O.D., 150 μm O.D. at the capillary inlet. Conditions: voltage 30 kV, current 32 μA. Detection: fluorescence, ex. 340 nm, em. 450 nm.

(the vacuolar subsample is injected into during the sample preparation process) should not exceed 1 nl, otherwise the complete mixing of the zone requires longer time, and diffusional zonebroadening may lead to a serious loss in efficiency. The exact conditions of on-column derivatisation are stated in the legend of Fig. 2.

3.3. Determination of sugars

The analysis of carbohydrates in picoliter volumes is a difficult task, as most carbohydrates contain neither easily chargeable nor chromophoric moieties. For the analysis of sugars in vacuolar samples a third approach had to be used, as none of the two previous methods is possible for the following reasons. The weak-acid behaviour of sugars (p*K* values around 12–13), requires highly alkaline conditions for their separation. This rather limits the use of indirect detection approaches for sugars, as according to

Garner and Yeung [49] high hydroxide concentrations lead to a decreased transfer ratio and therefore to rather limited sensitivity compared to other indirect systems. Precolumn derivatization is the most common strategy for carbohydrate detection. A wide variety of derivatization reagents is used for this purpose [50], and in most cases, reductive amination of the sugar carbonyl group is employed.

However, on-column derivatisation of sugars was not reported yet, as the reactions involved are slow (reaction time >1 h), often multi-step, and generally require rather high temperatures to proceed, conditions which are difficult to realise on-column. In addition, derivatisation of compounds which lack a free carbonyl group like sucrose, one of the most important metabolites in plants, is not possible.

Consequently, an alternative approach was used in this work, based on dynamic labeling. In dynamic labeling, the complexation of analytes with a complex partner leads to a change in UV absorbance or fluorescence. In the case of sugars chelation with Cu(II) provides in situ charged species that can be separated according to their different mobilities [45]. In addition a marked increase in UV absorptivity between 230 nm and 320 nm results from this complexation, which allows the direct detection of the chelates. As the background noise of the electrolyte is sufficiently low, the detection of micromolar concentrations of sugars is possible this way.

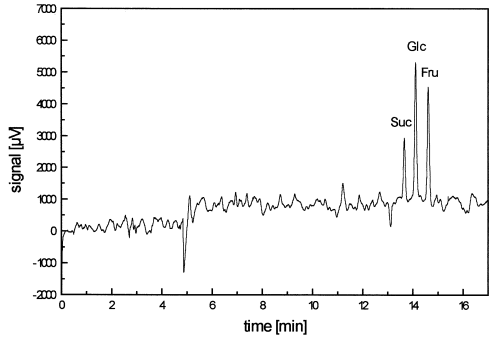

Fig. 3. Determination of sugars in a subsample of an epidermal vacuole of a wheat leaf using on-column chelation with Cu(II) and direct UV detection; the same constriction capillary for creation of the subsample was used as in Fig. 1. Peak: Suc = sucrose, Glc = glucose, Fru = fructose. Electrolyte: 7 m*M* copper(II) sulphate, 650 m*M* ammonia, pH 11.53. Capillary: 97 cm (74 cm to detector) × 50 μm I.D. × 360 μm O.D., 150 μm O.D. at the capillary inlet. Conditions: voltage 30 kV, current 18 μA. Detection: UV, 245 nm.

In Fig. 3 the detection of sucrose, glucose and fructose in a vacuolar sample obtained from a leaf-mesophyll cell of wheat using this dynamic labeling technique is shown. Again, the same constriction capillary was used as for the carboxylic acids, and internal standard addition became obsolete. As with the on-column derivatisation in the case of amino acids, the water droplet containing the vacuolar subsample should not be too large, as contrary to normal ion analyses, no efficient stacking process of the neutral sugars can take place, and thus unnecessarily high dilution factors must be avoided.

4. Conclusion

CE is a powerful method for the analysis of solutes and metabolites in individual plant cell vacuoles. Three fundamentally different methods for successful separation and detection of anions and carboxylic acids, amino acids and sugars have been presented, thus covering a large number of solutes and metabolites in vacuolar samples. Modification of the sample acquisition and handling procedure for vacuolar samples described in previous work is not necessary, since neither indirect detection, nor on-column derivatisation or dynamic labeling require any further sample pretreatment prior to injection into the CE capillary.

The high accuracy of the volumes sampled with constriction capillaries allows calibration of the latter using an internal standard in one of the CE systems. Once calibrated, a constriction capillary can be used for the creation of subsamples without the need of internal standardization, consequently sample handling is further reduced.

The described three methods allow the quantitative determination of a large number of components in single vacuoles and open the possibility of measuring concentration patterns between cells within plant tissue. In future work the determination of other species in vacuolar samples will be presented.

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