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Amino acid analysis in five pooled single plant cell samples using capillary electrophoresis coupled to laser-induced fluorescence detection

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Abstract

In this study 21 amino acid standards, samples of pure phloem sap and samples of pooled mesophyll cells were derivatized with fluorescein isothiocyanate, separated by capillary electrophoresis and detected with laser-induced fluorescence at 488 nm. Two different background electrolytes, a sodium borate buffer containing sodium dodecyl sulfate and a sodium borate buffer containing α-cyclodextrin, were used for the separation. Using the sodium dodecyl sulfate buffer, 14 amino acid standards could be separated, spiking identified 12 amino acids in pure phloem sap and 13 amino acids in pooled mesophyll cells. With the α-cyclodextrin containing background electrolyte, a resolution of 20 amino acid standards could be attained, 17 amino acids in pure phloem sap and 10 amino acids in mesophyll cells could be assigned. Leucine and isoleucine comigrated in both buffer systems. All separations were performed with a voltage of +20 kV and completed within 30 min. The detection limits obtained were in the fmol range for the sodium dodecyl sulfate and in the pmol range for the α-cyclodextrin background electrolyte. Compared to the one published capillary electrophoresis-based method for the determination of amino acids from few plant cells, the procedure described here allows very high sensitivity due to the use of laser-induced fluorescence detection and opens the possibility to dilute and measure pl samples with an fully automated, commercially available CE system.

Keywords: Plant materials; Background electrolyte composition; Amino acids

1. Introduction

Analysis of the chemical composition of bulk plant extracts provides only limited information, since the measured results are an average of all tissues present in the sample. Because several processes are defined on the level of a distinct tissue or even a single cell, techniques which allow the analysis of the chemical composition of cell types or even individual cells will enable new insights into the functional mechanism of plants.

Up to now most experimental approaches result in data, which average a couple of different cell types. For example leaves or leaf discs consist of at least three to four different tissues. Cell separating protocols like preparation of epidermal fragments, protoplasts or tissue peels do not represent cells in their intact, undamaged environment. A couple of approaches have emerged in the past few years which have overcome this problem by sampling a few or

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even single plant cells which are derived from the intact living plant by the use of glass microcapillaries [1,2]. The ability to sample and analyze the contents of single cells from intact plants is key since such analyses yield information from one tissue in one, and only one, developmental and physiological state. In an organ composed of various cell types, each cell type can be individually assessed [3]. One major problem in handling such small samples is the usually sub-nl volume (50–150 pl/cell), which can evaporate in seconds. However, a handful of methods have been applied successfully to the analysis of such very small samples, which contain minute amounts of the substances of interest. For example, ions can be analyzed by electron dispersive X-ray (EDX) analysis or ion selective electrodes [4] and sugars by microfluidic enzyme assays [5]. One major drawback of most of these protocols is that they are time consuming and labor intensive. In addition, simultaneous determination of several analytes is often not possible, which provokes interferences between sample components.

Capillary electrophoresis (CE) is a common tool for the analysis of a wide variety of substances, ranging from small inorganic ions to carbohydrates, proteins and nucleic acids [6]. Nouadje and coworkers [7,8] and Takizawa and Hakamura [9] also applied CE to amino acid analysis. The tested detection limit was in the fmol range and also detection limits in the amol range have been previously reported using fluorescence detection systems [10]. The available methods thus should be in theory sufficiently sensitive for the analysis of the amino acids in single cell samples. So far, most single cell studies using CE systems were performed on animal cells. For example, neurotransmitters, amino acids, cations, anions, and organic acids were qualitatively and partially also quantitatively analyzed ([11], reviewed in Ref. [12]). Recently, also some successful applications for the measurement of inorganic ions and organic acids in plant single cell extracts were published [2,13,14]. So far, only one method using CE for amino acid analysis at the single cell level in plants is available, which does not make use of the high sensitivity of laser-induced fluorescence (LIF) detection and a CE system with automated sample introduction [15]. Fully automated procedures could improve reproducibility, speed and ease of analyses.

In this report we describe a method in which a commercially available CE system with LIF and an automated sample introduction is applied to the analysis of amino acids in picoliter samples. To demonstrate the power of the method, we compared the amino acid content of two different cell types: the mesophyll as an example of amino acid synthesizing tissue and the phloem, which serves as a major route of amino acid transport from source to sink organs in plants.

2. Experimental

2.1. Plant material

Wild type pumpkin (*Cucurbita maxima* Duch. ''Gelber Zentner'') plants were grown in a growth chamber at an average photon flux density of 600 μmol m^{-2} s^{-1}. Temperatures were adjusted to 22°C, humidity to approximately 65%, and light to 14 h. Eight-to-12-week-old plants were used for the microsampling method as described below.

2.2. Sampling from mesophyll cells

Sampling of single plant cells was performed as described previously [2,3,5].

In short, borosilicate glass capillaries (WPI, Berlin, Germany) were pulled on a pipette puller (List, Darmstadt, Germany). Tip aperture was about 1 μm. The capillaries were mounted on a micromanipulator (Eppendorf, Hamburg, Germany) and cell samples were collected under a microscope (Nikon, Düsseldorf, Germany) with illumination from above. A capillary was inserted into a leaf still attached to the plant under optic control. After impalement into a cell, the turgor pressure forces a portion of mainly vacuolar cell sap into the capillary. To prevent contaminations from neighboring cells during the pass of the microcapillary through the leaf tissue, a positive pressure was applied to the end of the glass capillary. After impalement of the capillary into a target mesophyll cell, the pressure was released to let cell sap enter the capillary tip. The content of the microcapillary, usually containing a volume in the picoliter range, was then transferred to a 0.5-ml reaction vial containing 5 μl 0.2 *M* carbonate buffer. For the measurements of pooled mesophyll cells, the

contents of five single cells were collected into the same buffer vial.

2.3. Phloem extraction

A sterile needle was impaled into a fruit stem of a plant and phloem sap exudates collected from the wound site. The phloem sap was immediately released into a 0.5-ml reaction vial containing 50 μl chloroform to remove proteins and 100 μl 0.2 *M* carbonate buffer followed by centrifugation for 5 min at 12 000 rpm. The supernatant was derivatized as described below.

2.4. Derivatization procedure

Most amino acids show neither native UV absorption nor fluorescence. Therefore, a chemical derivatization with fluorescein isothiocyanate (FITC; Sigma, Deisenhofen, Germany) was necessary for detection. A $2.1 \cdot 10^{-6}$ *M* solution of FITC in acetone was prepared (dissolving 2.5 mg of FITC in 30 ml acetone followed by a 1:100 dilution with acetone). A 10 m*M* stock solution of each pure amino acid standard was prepared in 0.2 *M* carbonate buffer at pH 9.0 and used after appropriate dilution. A 50-μl volume of either each amino acid solution or the supernatant of the phloem extraction was allowed to react with 50 μl FITC solution for 2 h in the dark. For the blank samples 50 μl carbonate buffer was mixed with 50 μl FITC solution. The 5 μl buffer containing the pooled contents of five mesophyll cells were derivatized with an equal volume of FITC for 2 h in the dark.

2.5. Identification

Peaks were identified by spiking the purified phloem sap with $5 \cdot 10^{-6}$ *M* of one of each amino acid standards. Due to the small volumes and concentrations of pooled single cell samples spiking was not appropriate. The identification of the peaks in pooled single cell samples were performed by comparing the migration time of each amino acid standard with the migration time of the detectable peaks in the pooled single cell electropherogram.

2.6. Instrumentation and separation conditions

Separations were performed using a Beckman Coulter (Fullerton, CA, USA) P/ACE System MDQ 2000 associated with LIF (argon laser at 488 nm). The CE instrument was equipped with the P/ACE System MDQ software for instrument control and data handling. A 53 cm×50 μm I.D. fused-silica capillary (Chromatographie Service, Langerwehe, Germany) with an effective length to the detector of 42 cm was used for the separations. The polyimide coating at the detection window was burned off with a lighter.

Samples were stored at a temperature of 4°C during the measurements and the capillary was kept at 20°C. Two different running buffers were used for separation. The first running buffer consisted of 20 m*M* sodium dodecyl sulfate (SDS) and 100 m*M* boric acid, adjusted to pH 9.3 by addition of 0.1 *M* sodium hydroxide (NaOH). The second running buffer used for separation was 45 m*M* α-cyclodextrin (α-CD) in 80 m*M* sodium borate, pH 9.2. New capillaries were treated with 1 *M* NaOH for approximately 15 min before use. As a routine procedure before each sample injection, the capillary was rinsed with 0.1 *M* NaOH for 3 min at 20 p.s.i. followed by another 2 min at 20 p.s.i. with distilled water (1 p.s.i.=6894.76 Pa). Finally the capillary was equilibrated with the running buffer for 2 min at 20 p.s.i. Samples were injected by hydrodynamic injection for 5 s at 0.5 p.s.i. Separations were carried out by applying a potential of +20 kV.

3. Results

In this study, two different buffers were used for the separation of amino acids by CE–LIF detection using a conventional and automated Beckman CE system. Standards and plant samples derived from single cells or phloem elements, respectively, were extracted and their amino acid composition was analyzed. Two different background electrolytes (BGEs), one containing SDS, the other α-CD, were used for the separations.

Firstly, 20 proteinogenic amino acids and homoserine were derivatized and measured separately with the SDS BGE. Fourteen of the 21 amino acid standards could be resolved using the SDS

Table 1
Comparison of the separated amino acid standards and the determined amino acids in pure phloem sap and pooled mesophyll cells measured with both BGEs. Aminoacids with overlapping peaks were not included in the evaluation

Amino acid standard	Separated amino acids, SDS BGE	Separated amino acids, α-CD BGE	SDS BGE phloem pure	α-CD BGE phloem pure	SDS BGE pooled single cells	α-CD BGE pooled single cells
Alanine	+	+	+	+	−	+
Arginine	+	+	+	+	+	−
Asparagine	+	+	+	−	+	−
Aspartate	+	+	+	+	+	−
Cysteine	−	+	−	+	−	−
Glutamate	+	+	+	+	+	−
Glutamine	+	+	+	−	+	−
Glycine	+	+	+	+	+	+
Histidine	+	+	−	+	+	−
Homoserine	+	+	+	+	+	+
Isoleucine	+/−	+/−	+/−	+/−	+/−	+/−
Leucine	+/−	+/−	+/−	+/−	+/−	+/−
Lysine	+	+	+	+	+	+
Methionine	−	+	−	+	−	−
Phenylalanine	−	+	−	+	−	+
Proline	−	+	−	+	−	−
Serine	+	+	+	+	+	+
Threonine	+	+	+	+	+	+
Tryptophan	+	+	−	−	+	−
Tyrosine	−	+	−	+	−	+
Valine	−	+	−	+	−	+

(+) Amino acids, separated and determined; (−) amino acids, not separated and determined; (+/−), isoleucine and leucine coeluted.

BGE. Leucine and isoleucine coeluted. For the amino acid standards cysteine, threonine and serine two peaks were observed (see Table 1).

Samples of pure phloem sap were measured after purification and derivatization and several peaks could be observed in the electropherograms (Fig. 1). For the identification of peaks the samples were spiked with a known concentration of each amino acid standard. Fig. 1 shows an electropherogram of phloem spiked with $5 \cdot 10^{-6}$ *M* threonine, aspartate or glutamate, respectively, in comparison to pure phloem sap using the SDS BGE. Using this approach, 12 of the 21 tested amino acids could be discriminated in pure phloem sap (Fig. 2). Amino

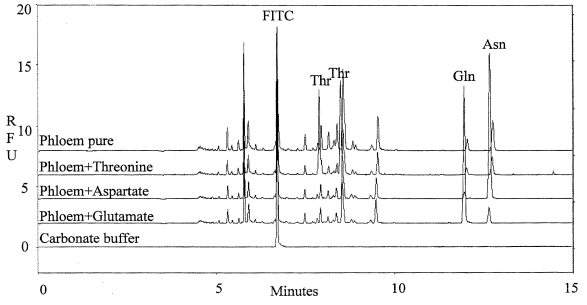

Fig. 1. Electropherograms of pure phloem sap, phloem spiked with amino acid standards, and carbonate buffer as a blank. The samples are labelled with FITC at $2.1 \cdot 10^{-6}$ *M*. Migration conditions: 20 m*M* SDS, 100 m*M* borate, pH 9.3, +20 kV. All peaks are normalized to the FITC peak. RFU=Relative fluorescence units.

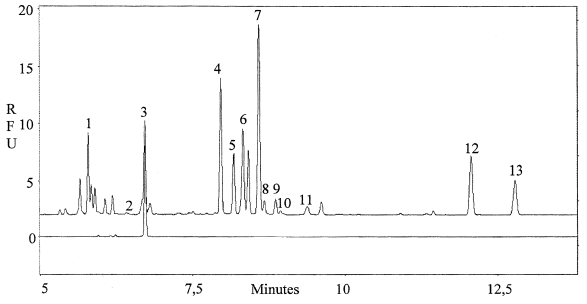

Fig. 2. Electropherogram of pure phloem sap. Electrophoretic conditions: see Fig. 1. (1) Arginine, (2) lysine, (3) FITC, (4) threonine, (5) leucine/isoleucine, (6) glutamine, (7) homoserine, (8) asparagine, (9) serine, (10) alanine, (11) glycine, (12) glutamate, (13) aspartate. The lower line represents the electropherogram for carbonate buffer as a blank.

acids, which showed overlapping peaks were not included in the evaluation. Some peaks present in the phloem samples could not be identified by adding amino acid standards.

To overcome the problem of overlapping peaks, we tested a second buffer system containing α-CD, which is known to enhance the selectivity of the separation. Twenty amino acid standards were well separated, but the pair leucine and isoleucine could not be resolved. A higher volume (6 μl) of phloem sap was used for measurements with this buffer system. In phloem sap, 17 amino acids could be identified using α-CD as a BGE (Fig. 3). Similar to the results obtained with the other buffer system, there were some additional peaks in the electropherograms that could not be identified by spiking in amino acid standards.

Table 1 presents the amino acids which were detected using the two different BGEs. The order in which some of the amino acids, for instance threonine, alanine and glycine, migrated was different for the two running buffers (compare Fig. 2 and Fig. 3). In addition, some amino acids, which were detectable with the SDS BGE in the biological samples (glutamine, asparagine), could not be detected with the α-CD BGE and vice versa (e.g., cysteine and phenylalanine).

For amino acid determination in the second cell type, single mesophyll cells were sampled by glass microcapillaries, five extracts were pooled and subsequently analyzed with respect to their amino acid content. The SDS BGE revealed several peaks. Thirteen of them could be identified by comparing the migration times (Table 1, Fig. 4). Also here, some peaks could not be identified. When analyzed with the α-CD BGE, the mesophyll samples showed fewer peaks, which suggests a lower sensitivity of the BGE system. Ten different amino acids could be discriminated in this cell type using α-CD as BGE (Fig. 5).

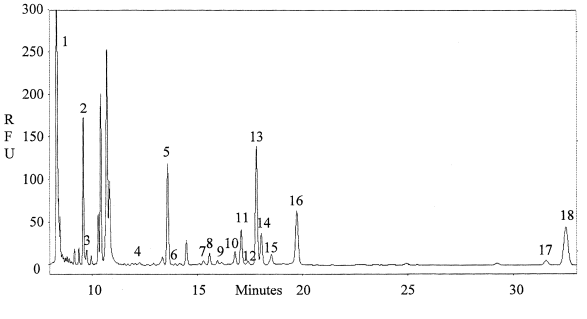

Fig. 3. Electropherogram of pure phloem sap. Migration conditions: 45 m*M* α-CD BGE, 80 m*M* borate, pH 9.2, +20 kV. (1) FITC, (2) arginine, (3) lysine, (4) cysteine, (5) leucine/isoleucine, (6) methionine, (7) phenylalanine, (8) threonine, (9) tyrosine, (10) valine, (11) proline, (12) histidine, (13) homoserine, (14) serine, (15) glycine, (16) alanine, (17) glutamate, (18) aspartate.

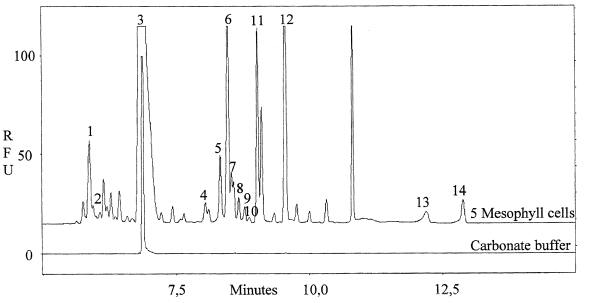

Fig. 4. Electropherogram of pooled mesophyll cells. Electrophoretic conditions: see Fig. 1. (1) Arginine, (2) lysine, (3) FITC, (4) threonine, (5) leucine/isoleucine, (6) glutamine, (7) histidine, (8) homoserine, (9) asparagine, (10) tryptophane, (11) serine, (12) glycine, (13) glutamate, (14) aspartate.

4. Discussion

The present study compares two different background electrolytes and their applicability to amino acid analysis at the level of a few pooled single cell samples. In contrast to previously published procedures [7], in which the samples were diluted with

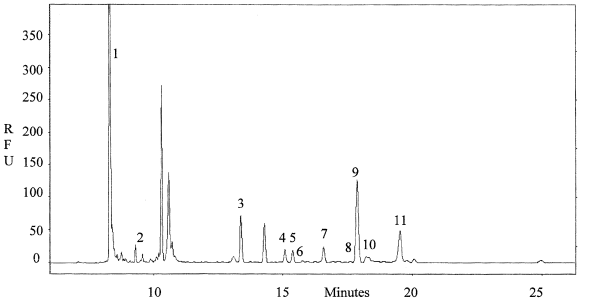

Fig. 5. Electropherogram of pooled mesophyll cells. Migration conditions: see Fig. 3. (1) FITC, (2) lysine, (3) leucine/isoleucine, (4) phenylalanine, (5) threonine, (6) tyrosine, (7) valine, (8) homoserine, (9) serine, (10) glycine, (11) alanine.

distilled water after derivatization, the samples were analyzed directly after derivatization with a $2.1 \cdot 10^{-6}$ *M* solution of FITC, because volume and concentration of pooled single cells were too small to allow supplementary dilution.

By means of SDS BGE, 14 amino acid standards were separated by micellar electrokinetic chromatography (Table 1). In contrast to this study, a separation of tyrosine, valine and phenylalanine in cerebrospinal fluid, but a failure to detect homoserine or asparagine has been previously reported in the literature [7]. This may be due to the nature of the biological samples analyzed.

Two peaks were found for the amino acid standard cysteine, (data not shown). Because cysteine is easily oxidized on exposure to air, cystin, a cysteine dimer connected by a disulfur bridge is formed and could explain this second peak [16]. A direct coupling between a CE system and a mass spectrometer could help to determine the nature of the two peaks [16]. Two peaks were also observed for the amino acid standards threonine (Fig. 1) and serine, though the reason for this is unclear.

To overcome the problem of overlapping peaks, a second background electrolyte containing α-CD was used. All of the 21 amino acid standards, except of isoleucine and leucine, could be separated (Table 1). α-CD consists of six glucose units and can enhance the selectivity of CE [17]. Selectivity is taken to be a function of whether and how the amino acids interact with the α-CD hydrophobic cavity [17]. Whereas the separation could be improved for most amino acids with α-CD, isoleucine and leucine comigrated in both buffer systems, which is most probably due to their very similar structures and molecular masses. A separation could be achieved by employing a β- instead of α-CD BGE [17].

A comparison of both buffer systems showed that the measurements using the α-CD BGE required more time, but the resolution was higher (Fig. 2, Fig. 3). In contrast, the SDS BGE has the advantage of a higher sensitivity [9] and therefore less sample had to be taken for the measurements. Using the described method, the detection limit for the SDS BGE was in the fmol range, while the α-CD BGE had a detection limit in the pmol range. That means that with the injection and separation conditions applied, sample concentrations in the nanomolar range for the SDS BGE and in the micromolar range for the α-CD BGE are required. In addition, the order in which some amino acids migrated was different between both BGEs, for example threonine, alanine and glycine (compare Fig. 2 and Fig. 3).

Samples of pure phloem sap were measured with both running buffers. The electropherograms of the phloem sap revealed many peaks and 12 out of the 21 tested amino acids could be identified using the SDS BGE (Fig. 2). Seventeen amino acids were identified with the α-CD BGE (Table 1, Fig. 3).

The spiking method did help to identify the peaks, but not all of the peaks were found to correspond to one of the 21 amino acids tested. On the other hand also not all 21 amino acids could be detected in the phloem sap (Fig. 1). It may be that not all of the tested 21 amino acids are present in the phloem samples in sufficiently high concentrations. A comparison of the identified amino acids in pure phloem sap (Table 1) with the amino acid composition measured previously in phloem of *Cucurbitaceae* showed that the same amino acids could be identified with the CE procedure for small volumes described here [18]. The relative concentrations of the different derivatized substances in phloem sap varied from sample to sample. Such variation is likely attributable to developmental stage, physiological state of the plant or even time of sampling. The large variation in the relative concentration of homoserine was especially noticeable. A comparison of the two cell types revealed some differences in their amino acid composition. Several peaks occurred in the electropherogram of both cell types. Some peaks present in the samples could up to now not be identified as one of the 21 tested amino acids. The identity of these ''unknowns'' remains unclear because the derivatization reagent is not only specific for amino acids, but also for compounds having primary amino groups (e.g., unusual amino acids, amino sugars, amino alcohols, katecholamines, amines and peptides).

The pooled single cell samples were also measured with both BGEs (Fig. 4, Fig. 5). Thirteen amino acids could be detected in pooled single cells using the SDS BGE (Fig. 4). Bazzanella et al. [15] identified nine amino acids derivatized with *o*-phthaldialdehyde–2-mercaptoethanol (OPA–2-ME) in single cells from wheat by using CE associated

with fluorescence detection at 340 nm. Among others they could detect valine, alanine and methionine, which were not detectable in pooled single cells measured with the SDS BGE used in this study. Alanine and valine were detectable with the α-CD BGE (Table 1). By using LIF detection and FITC as the derivatization reagent seven additional amino acids could be determined in samples of pooled single cells using the SDS BGE, which were not found previously [15]. In addition, with the α-CD BGE phenylalanine, tyrosine, alanine and valine were identifiable in samples of pooled single cells, but not glutamate or aspartate (Table 1). Reasons might be differences in the amino acid content of plant species or different derivatization efficiencies for amino acids of the two reagents. Another major improvement is the use of a commercial CE system, which is fully automated and equipped with an autosampler. This avoids labor-intensive and time consuming sample injection with optic control under a microscope.

5. Conclusions

The described method makes use of a commercial available fully automated CE system which is equipped with a highly sensitive LIF detector. The described method allows the determination of a large number of amino acids in phloem and pooled single cells and opens the possibility to compare different cell types within undamaged plants.

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