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Determination of ascorbic acid in isolated pea plant cells by capillary electrophoresis and amperometric detection

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Abstract

A capillary electrophoretic method for the determination of ascorbic acid in a single protoplast, i.e., a plant cell without cell wall, has been developed. Amperometric detection using a carbon fibre disc electrode at 1.0 V vs. Ag/AgCl was applied. The running buffer consisted of 10 m*M* phosphate, pH 7. The limit of detection was approximately 5 μ*M* for measurements on single protoplasts, typically 20 μm in diameter, and 22 n*M* for measurements with ascorbic acid standard solutions. The concentration of ascorbic acid varied between 0.05 and 1.6 m*M* for five selected, isolated and injected protoplasts.

Keywords: Ascorbic acid

1. Introduction

Ascorbic acid (AA) is a major and universal metabolite in photosynthetic eukaryotes. In most plant tissues, millimolar concentrations can be found. The function and metabolism of AA in plants has recently been reviewed [1]. AA supports many functions, such as acting as an antioxidant, constituting an enzyme cofactor, transporting electrons and being a precursor in oxalate and tartrate synthesis. These functions are primarily based on the ability of AA to act as a reversible biological reductant. AA is widely distributed in plant cells. Its presence in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall has been shown [1].

Capillary electrophoresis (CE) is a highly efficient separation technique requiring injection volumes in the nanoliter range or less. Coupled to laser induced fluorescence (LIF) or amperometric detection, highly sensitive and selective detection is achieved. Consequently, it has gained in popularity in the area of single cell analysis, thereby enabling functional studies of specific cells and their response to external stimuli. Several single cell methods for the determination of various compounds, ranging from small inorganic anions to high-molecular-mass proteins, in animals, have been presented [2–11]. An excellent review on the subject has been presented by Jankowski et al. [12].

To date, however, application of the technique to plant cells has been poorly exploited. Some pioneering work has been performed by Honda et al., who determined AA and some other components in single parenchymatous cells of citrus fruit with CE and UV detection [13]. Hydrodynamic injection was per-

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formed with the 50 μm I.D. separation capillary directly inserted into the 17 μl cell. Further, Bazzanella et al. determined inorganic cations and anions in 20 to 60 pl extracted vacuolar sap volumes from the epidermis of a wheat leaf [14].

With respect to the determination of ascorbate in plant tissues and related samples, such as tea or juice, several other methods have been used based on CE and utilising UV [15–23] or indirect UV detection [24]. Among these methods, the lowest detection limit reported is 2 μ*M* [24]. An obvious way to improve detection limits, and thereby decrease the required sample volumes, would be to use amperometric detection [2].

In this work, the determination of ascorbate in single plant cells with CE and amperometric detection is demonstrated. The method, which was applied to protoplasts isolated from leaves from pea (*Pisum sativum*), is suitable for cells with volumes in the $5 \cdot 10^{-12}$ l range.

2. Experimental

2.1. Capillary electrophoretic system

Untreated fused-silica capillaries, 67–72 cm×20 μm I.D., 150 O.D., were obtained from Polymicro Technologies, (Phoenix, AZ, USA). Before use, the capillaries were washed with 0.1 *M* NaOH for 30 min, deionized water for 20 min, and operating electrolyte for 20 min.

The input of the high-voltage power supply, ±0–30 kV, (Brandenburg, Thornton Heath, UK), together with the injection end of the capillary, were placed in a Plexiglas box with an interlock on the access door for protection. Rinsing of the capillary was accomplished by applying pressure on the injection buffer. Injections of standards were made hydrodynamically by raising the injection side 2 cm for 4 min, resulting in an injected volume of 0.46 nl. End-column amperometric detection was performed using a three electrode configuration. The potentiostat used was an LC-4B amperometric detector (BAS, West Lafayette, IN, USA). Data acquisition was accomplished with a PC equipped with a PCL-812PG lab card (Advantech, Taiwan), and a laboratory-developed QBASIC computer program. An ELDS 900 laboratory data system (Chromatography Data Systems, Kungshög, Sweden) was used for integration.

The detector cell has been described in detail previously [25]. The working electrode consisted of a 30-μm carbon fibre disk. As reference, a Ag/AgCl electrode in 3 *M* KCl gel (BAS) was used, and the auxiliary electrode was a platinum wire with a diameter of 2 mm (Goodfellow, Cambridge, UK). The working electrode was daily washed with bichromate–sulfuric acid for 30 s, and then with deionized water. It was positioned directly in front of the capillary outlet, at a distance of approximately 5 μm. This was accomplished by adjusting the working electrode with a micropositioner under the view of a microscope. The detection and injection arrangements were accommodated in Faraday cages. The electroosmotic flow (EOF) was identified by injection of butulated hydroxytoluene.

2.2. Etched capillaries

To facilitate the cell injections, the injection side of the capillary was etched to a conical shape [26]. Approximately 2 mm capillary was placed in 40% hydrofluoric acid (Aldrich, Steinheim, Germany), and flushed with nitrogen at a pressure of 1.5 bar for 15 min. The capillary was then washed in concentrated sodium carbonate and deionized water. A 5-mm length of the polyimide coating was immediately removed by burning. The capillary tip was shaped with a scalpel and etched for an additional 10 min. This resulted in an O.D. of 75–100 μm and an I.D. of 50–75 μm at the capillary entrance.

2.3. Chemicals and preparation of solutions

All chemicals were of analytical grade, and solutions were prepared using water purified with an Elgastadt UHQ II system (Elga, UK). A standard solution of 1 m*M* ascorbic acid was prepared daily. The running buffer consisted of 10 m*M* phosphoric acid adjusted to pH 7.0 with sodium hydroxide.

Enzyme solution: 0.5 *M* sorbitol, 1.3% cellulase (1.1 U/mg) (Serva, Heidelberg, Germany), 0.3% macerase (0.52 U/mg) (Serva), 0.05% bovine albumin (A 8022, Sigma, St. Louis, MO, USA), 0.05% polyvinylpyrrolidone (average molecular mass 10 000), 20 m*M* 2-morpholinoethanesulfonic acid (MES), pH 5.5, 1 m*M* $CaCl_2$.

Medium 1: 0.5 *M* sorbitol, 1 m*M* $CaCl_2$, 5 m*M* N-[2-hydroxyethyl] piperazine-N-[2-ethane sulfonic acid] (HEPES), pH 7.0.
Medium 2: 0.5 *M* sucrose, 1 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.0.
Medium 3: 0.1 *M* sorbitol, 0.4 *M* sucrose, 1 m*M* $CaCl_2$, 5 m*M* HEPES, pH 7.0.

2.4. Isolation of protoplasts

Plant cells differ from animal cells in that they are surrounded by a cell wall mainly consisting of cellulose. In order to be able to inject the plant cells into the CE system and to lyse them, this wall had to be removed. This was performed by applying an enzymatic digestion procedure resulting in protoplasts, plant cells without a cell wall. The whole protoplast isolation procedure, a slightly modified version of the method described by Dodds [27], also served as a purification step, since extracellular ions were removed.

Seeds of pea (*Pisum sativum* L. cv Marma) were grown in trays with vermiculite in a growth chamber. After 10 days, about 0.25 g of fully developed leaves were cut into 1-mm segments, and placed in 10 ml of the enzyme solution in a beaker. The beaker was kept in a water bath at 30°C for 3 h. The enzyme solution was then removed with a Pasteur pipette and replaced by 2.5 ml of medium 1. The beaker was shaken, very gently, a few times in order to release the protoplasts from the cut surfaces. The medium, now containing a large number of protoplasts, was filtered through a 100-μm nylon filter in order to remove large pieces of plant material. This procedure was repeated with an additional 2.5 ml of medium 1. The resulting 5 ml of filtrate was centrifuged at 165 *g* for 5 min. The pellet was suspended very gently in 1 ml of medium 2. Carefully, 0.5 ml of medium 3, and then 0.5 ml of medium 1, were layered on top. The created sugar gradient was then centrifuged at 80 *g* for 4 min. The protoplasts, that formed a layer between media 1 and 3, were removed with a Pasteur pipette.

2.5. Protoplast injection

A 50-μl portion of the protoplast suspension was placed on a microscope slide under a stereo microscope (60× magnification, Nissho Optical, Japan). Before analysis, the cell suspension was mixed with 50% of medium 1. This facilitated the injection procedure since the protoplasts sedimented to the bottom. Further, 1 μl of 0.1 *M* HCl was added in order to neutralise the negatively charged protoplasts. Otherwise the protoplasts were repelled from the capillary entrance, migrating towards the positive electrode during the injection.

The high-voltage Pt electrode was placed in the cell suspension droplet together with the injection end of the capillary, which was mounted on a 3D micromanipulator. This allowed the capillary to be placed in front of the selected cell. Then, an injection voltage of 3 kV was applied until the cell had been forced about 10 μm into the capillary by the EOF produced (usually after 5–30 s). Afterwards, the capillary and the Pt electrode were placed back into the running buffer in the plexiglass box. When the separation voltage of 30 kV was applied, the protoplast disrupted due to the osmotic shock, and possibly also by the sudden electric pulse produced.

3. Results and discussion

3.1. Calibration data for prepared ascorbic acid solutions

A buffer of 10 m*M* phosphate, pH 7.0, was chosen as running buffer. The limit of detection (LOD) for AA dissolved in media 1 and 2 (1:1) was found to be 22 n*M* (10 amol), calculated from three times the noise. This is at least 100 times better compared to other methods where UV and indirect UV detection is performed [15–24]. R.S.D. values for five hydrodynamic injections of 20 μ*M* AA were 4.1% for the area and 1.5% for the migration time. The calibration curve was linear between 50 and 20 μ*M* (r^2=0.999, n=5). No effects on migration time, peak shape or area were observed when standards were prepared in media 1 and 2 compared to preparation in water.

3.2. Single cell analysis and identification of ascorbic acid

In Fig. 1, an electropherogram from injection of a protoplast is displayed. Injections of the suspension medium surrounding the protoplasts and pure medium were made in order to be able to trace the

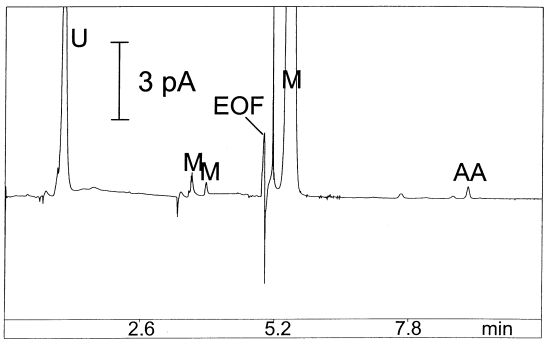

Fig. 1. Electropherogram of a protoplast lysed in the capillary. The peaks originating from the cell medium are marked M, the ascorbic acid peak AA and the electroosmotic flow is denoted EOF. Amperometric detection at a 30-μm carbon fibre disc electrode, 1.0 V vs. Ag/AgCl; buffer: 10 m*M* phosphate, pH 7.0; capillary: 67 cm×20 μm I.D.; separation potential: 30 kV.

Table 1
Calculated mobilities for peaks in protoplast run No. 3 (see Fig. 2, No.3) and for the AA peak in a standard

Peak No.	Calculated mobility ($m^2\ s^{-1}\ V^{-1}$)
1 in run No. 3	$-1.89\cdot10^{-8}$
2 in run No. 3	$-2.09\cdot10^{-8}$
3 in run No. 3	$-2.29\cdot10^{-8}$
4 in run No. 3	$-2.40\cdot10^{-8}$
5 in run No. 3	$-2.48\cdot10^{-8}$
AA in standard	$-2.41\cdot10^{-8}$

Conditions as in Fig. 1.

origins of the peaks. The peaks found in the pure media were marked with M in Fig. 1. No peaks originating from the possible release of intra-cellular compounds into the medium were found. The large peak, denoted U (unknown), appeared following analysis of cell Nos. 3 and 4, but for none of the other cells. Portions of the electropherograms obtained from injections of five different protoplasts are displayed in Fig. 2. Protoplast Nos. 1 and 2 are two consecutive injections from the same protoplast suspension, whereas Nos. 3–5 are three consecutive injections from a suspension prepared 2 weeks later. In both cases the plants were 10 days old. The pattern of the peaks from all the protoplasts is very similar. Sometimes large shifts in the EOF and migration times were observed, probably due to adsorption of constituents of the cell onto the capillary wall and/or due to different amounts of acidified medium introduced into the capillary during the injection procedure. AA was identified by calculating the electrophoretic mobility for the peaks found in the electropherograms originating from the cell injections and comparing them with the mobility of the AA standard peak obtained according to:

$$\mu = \frac{L^2(t_m^{-1} - t_{EOF}^{-1})}{V}$$

where L is the length of the capillary, t_m and t_{EOF} are the migration times of the peak and the EOF, respectively, and V is the applied voltage. This is exemplified for cell No. 3 in Table 1.

3.3. Quantification of ascorbic acid

In Table 2, the results from semi-quantitative determinations of AA in five different protoplasts are presented. The calculations were based on one point calibrations from a 10 μ*M* AA standard run performed immediately before the cell injections. Peak areas were corrected for differences in speed when migrating past the detector. The volumes of protoplasts are easy to calculate, if their radii can be

Table 2
Concentrations of AA in five different protoplasts

Cell No.	Diameter (μm)	AA amount (fmol)	AA conc. in cell	
			μg/g fresh mass	m*M*
1 (day 1)	20	0.2	9	0.05
2 (day 1)	30	23	30	2
3 (day 2)	20	1.5	60	0.4
4 (day 2)	20	1.2	50	0.3
5 (day 2)	20	2.3	100	0.6

Conditions as in Fig. 1.

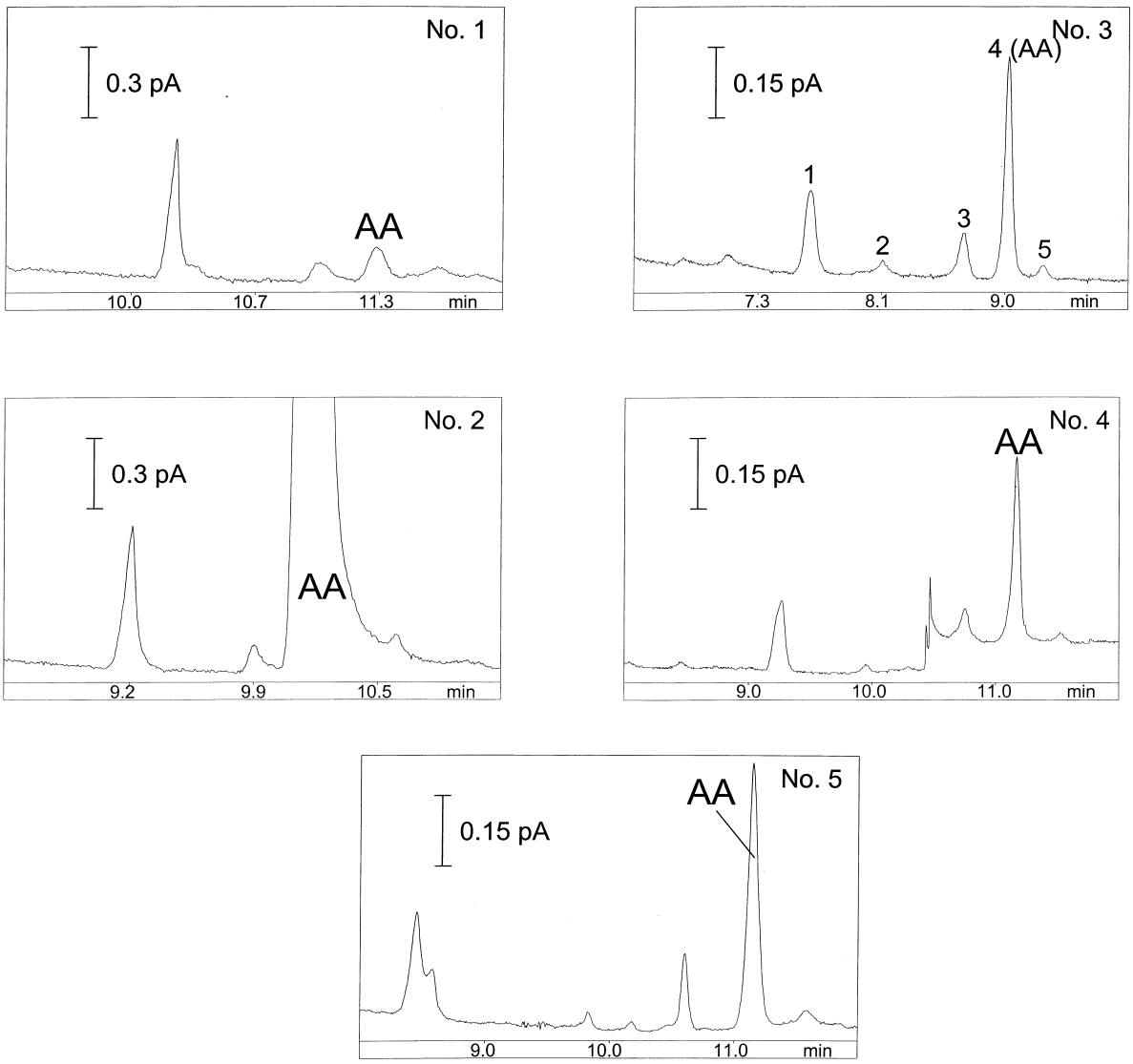

Fig. 2. Electropherograms of five different protoplasts; AA, ascorbic acid. Protoplasts 1 and 2 originate from the first protoplast suspension (prepared day 1). For these, conditions were as in Fig. 1, except the capillary length: 72 cm. Protoplasts nos. 3–5 originate from a protoplast suspension prepared another day (day 2). Conditions as in Fig. 1.

measured accurately, because of their almost perfectly spherical shape. However, the diameters were roughly estimated by a comparison with a 20-μm object. Therefore, the calculated cell volumes, and thereby the concentrations of AA in the protoplasts, can be subject to an error of almost 40%. By assuming that the density of the cells is 1 g/ml, AA values ranging from 9 to 100 μg/g fresh mass are obtained (Table 2). Considering that the total amount in extracts from the leaves of pea have been determined by HPLC to be 230 μg/g fresh mass [28], the values seem reasonable.

Protoplast No. 2 (Fig. 2) was larger and more transparent than the other protoplasts. In this cell, the concentration of AA was considerably higher than in the other cells, especially relative to the other peaks

appearing in the electropherogram. Possibly, this is a different type of cell supporting a different function in the plant. The amount of AA is known to vary substantially between different tissues in the plant, often the higher levels are found where a rapid development is taking place [1].

It is not clear how the isolation of the protoplasts, and their acidification before injection, will affect the AA concentrations. Plant stress or disturbances to the metabolism are known to activate enzymes, and, usually, an altered AA concentration is observed [29,30]. Since the protoplast isolation begins with causing serious damage to the plant, it is possible that the AA concentrations measured are anything but typical.

3.4. Concluding remarks

The present work shows that the determination of AA in a single plant cell is possible using CE with amperometric detection. By removing the cell wall with an enzymatic treatment procedure, the whole resulting protoplast can be injected, and the total amount of internal cellular substances can be determined. Problems associated with extraction efficiencies and the instability of AA during sample preparation are no longer present. However, the protoplasts cannot be directly compared with cells in a tissue, since they are removed from their natural environment. The surrounding tissue plays an important role in dictating the cells' function. Also, the cell wall and the enzymes bound therein, and other compounds are missing.

The single plant cell analysis previously described where vacuolar sap is injected either by inserting the capillary directly into the cell [13] or by injection of withdrawn sap [14] is advantageous since it allows better control of the injection volume. Thereby the concentration determination becomes more accurate. In addition, a specific cell can be selected directly in the tissue. However, these methods become more difficult to apply as cell volumes gets smaller. By applying the method described in this work the whole cell, except the cell wall, is injected, and effects of extracellular influences might easily be studied by exposing the protoplasts to different treatments. It should also be possible to sort out rare cell types and cells in different stages of development, clarifying their specific roles. The electropherograms obtained reflect the redox status of the injected cell. Therefore this method should be relevant in studies of, for instance, oxidative stress in plant tissues.

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