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Short communication

Monitoring of the purification of systemin by capillary electrophoresis

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Abstract

Capillary electrophoresis (CE) was applied as a method for monitoring of the separation process of systemin, an endogenous peptide from tomato plants. Systemin, an 18-amino-acid polypeptide, was isolated from tomato leaves and purified. A systemin peak was identified using synthetic systemin as a marker, and traced by CE and reversed-phase high-performance liquid chromatography (RP-HPLC) during all chromatographic steps. The best results of CE analysis were obtained in 25 m*M* phosphate buffer at acidic pH using a constant operating voltage of 30 kV.

Keywords: Systemin; Peptides

1. Introduction

Biochemical studies of plant defence responses have led to the discovery of several molecules functioning as potential systemic signals to pathogens and pests [1]. In 1991 an 18-amino-acid endogenous polypeptide (called systemin) was isolated from young tomato leaves [2]. The sequence of systemin is a palindrome [2]: Ala^{1}–Val–**Gln**–Ser–**Lys – Pro – Pro** – Ser – Lys – Arg^{10} – Asp – **Pro – Pro**–**Lys**–Met–**Gln**–Thr–Asp^{18} (amino acids creating a palindrome are in boldface). Systemin is the only plant polypeptide hormone-like signaling molecule presently known. It activates the expression of genes encoding proteinase inhibitors I and II in response to insect or pathogen attack, or other severe wounding of tissues [2]. The latest data show that systemin has a much broader spectrum of activity than previously known. Systemin not only regulates the synthesis of proteinase inhibitors but also activates synthesis of polyphenol oxidase (PPO) and 1-aminocyclopropane-1-carboxylate synthase (ASS), the key enzyme in ethylene biosynthesis [3,4]. Both enzymes are probably involved in plant-resistance mechanisms. Systemin is a potent inducer of alkalization response [4]. The decrease of H^+ concentration in extracellular space is coupled to an increase of K^+ concentration. A role for this systemin activity is unknown.

Systemin was analyzed previously by RP-HPLC on a octadecylsilane (C_{18}) column and by strong cation-exchange HPLC (SCX-HPLC) on a poly-sulfoethyl aspartamide column [2,5]. In both cases satisfactory separation was achieved but the retention time was relatively long (10-min range). Here we

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show that capillary electrophoresis (CE) is useful for monitoring of the isolation process of systemin in the range of a few min.

2. Experimental

2.1. Plant material and preliminary extraction

Tomato plants were grown without any control conditions. Leaves of individual plants were harvested and 0.5 kg of green leaves were collected. The leaves were homogenized in 1.5 l of distilled water for 10 min and centrifuged at 750 *g* for 3 min. The supernatant was decanted and adjusted to pH 3 with 6 *M* HCl and centrifuged again under the same conditions. The solution was adjusted to pH 7 with 6 *M* NaOH and centrifuged at 1700 *g* for 10 min. The brown-green solution was filtered through a 0.45-μm membrane filter, frozen, lyophilized and 22.5 g of grey-brown solid material was obtained.

2.2. Chemical synthesis of systemin

Systemin was synthesized using Boc (*tert.*-butyloxycarbonyl) chemistry, by the solid-phase method on a cross-linked polystyrene resin substituted with Boc–Ala (0.68 mmol/g of resin). Following cleavage by hydrogen fluoride (HF), the crude peptide was purified by gel chromatography and C_{18} RP-HPLC. Details will be published elsewhere [6].

2.3. Methods

Fractions with the highest contents of systemin were combined, frozen and lyophilized.

2.3.1. Gel filtration

Crude tomato leaf extract was filtered on a Sephadex G-25 column (105×2.8 cm) in 30% acetic acid. Detection was performed at 226 nm. Flow-rate was 30 ml/h. The crude extract (22.5 g) was loaded onto a column and 1 g was obtained. After ion-exchange chromatography, fractions with the highest purity were desalted on a Sephadex LH-20 column (30×1.5 cm) in 30% acetic acid. Filtration was monitored at 226 nm, the flow-rate was 10 ml/h. 0.5 mg of the sample obtained after ion-exchange chromatography was loaded onto a column and 100 μg was obtained.

2.3.2. Preparative high-performance liquid chromatography

Preparative RP-HPLC was performed using a Vydac C_{18} column (24×3.2 cm, 15–20 μm particle size). Solvent system: (a) 0.1% trifluoroacetic acid (TFA), (b) 15% acetonitrile. A linear gradient from a to b for 1.5 h was used. The separation process was monitored at 226 nm. Flow-rate was 16 ml/min. After Sephadex G-25 chromatography about 1 g of material was loaded onto a column and 2 mg was obtained.

2.3.3. Ion-exchange chromatography

A sample was separated on a Sepharose S Fast Flow column (13×1.5 cm). A linear gradient was used. Eluents were: (a) 10 m*M* ammonium formate–20% ethanol, pH 3.5, (b) 10 m*M* ammonium formate–20% ethanol–500 m*M* NaCl, pH 3.5. Detection was performed at 226 nm. Flow-rate was 19 ml/h. Of the material after RP-HPLC chromatography 2 mg was loaded onto a column and 500 μg was obtained.

2.3.4. Capillary electrophoresis

A Beckman P/ACE System 2100 capillary electrophoresis instrument with the cathode on the detection side was employed. All solutions and samples were filtered through a 0.22-μm PTFE membrane filter prior to use. Electrophoresis was performed in a 25 m*M* phosphate buffer at pH 2.5. The capillary cassette used was fitted with a 75 μm I.D. uncoated fused-silica capillary, 57 cm in length (50 cm to the detector). Runs were made at a constant voltage of 30 kV. Temperature of the capillary was maintained at 25±0.1°C. The separation effect was monitored at 214 nm. The chromatography data was acquired and stored on an IBM 386 DX-40 computer using the System Gold software package. Data was exported as DIF (data interchange file) to the Sigma Plot program and plotted.

2.3.5. Analytical high-performance liquid chromatography.

Analytical RP-HPLC analysis was performed using a Beckman System Gold Chromatograph with

an Ultrasphere ODS column (19.5×0.46 cm, 5 μm particle size). Solvent system: (a) 0.1% TFA, (b) acetonitrile–0.1% TFA (80:20, v/v). A linear gradient of 5–25% b for 20 min was used. Flow-rate was 1 ml/min, absorbance at 226 nm.

2.3.6. Amino-acid analysis

The analysis was performed on a Beckman Model 121M analyzer. A sample of systemin was hydrolyzed with constantly boiling HCl containing 1% of phenol at 110°C for 24 h.

2.3.7. Amino-acid sequencing

The sequence was determined on an Applied Biosystems sequencer, Model 473A. The amino acids were determined as phenylthiohydantoin (PTH) derivatives.

2.3.8. FAB-MS

The sodium salt of the molecule was determined using the fast atom bombardment method (FAB) with a AMD-604 double focusing mass spectrometer with BE geometry. The ion source was equipped with a Cs^+ gun, energy of the ions was 12 keV. Analysis was performed in a glycerine matrix.

3. Results and discussion

Because of the complex composition of biological material obtained from tomato leaves and the small

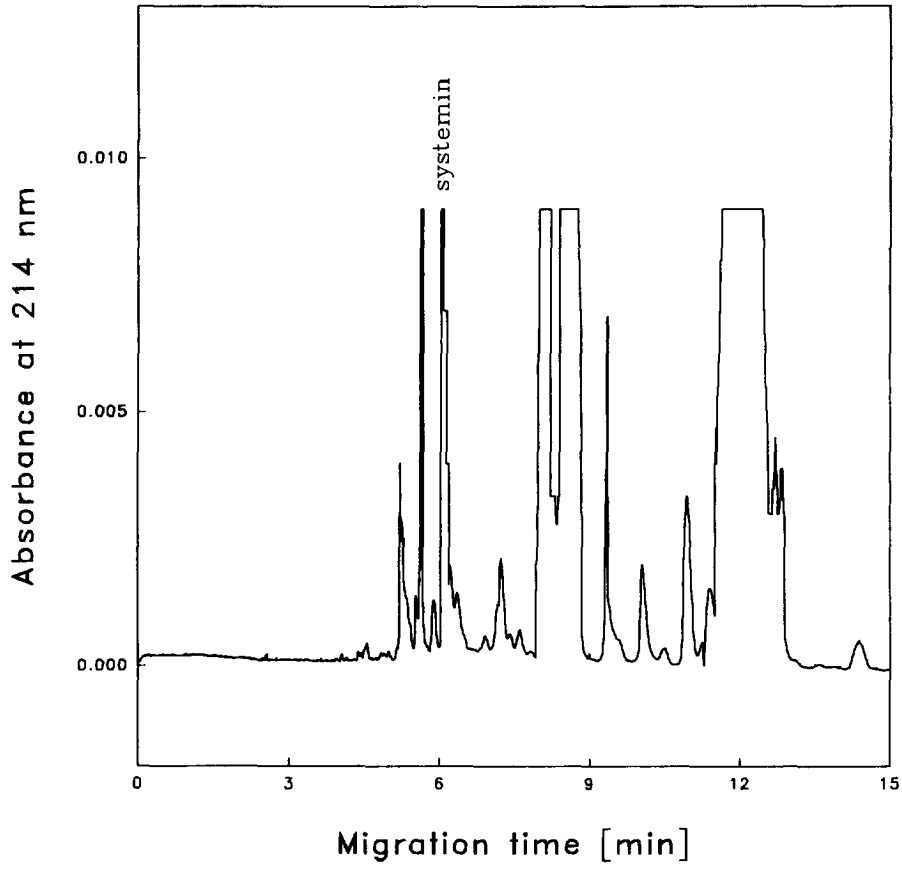

Fig. 1. CE analysis of crude tomato leaf extract. Electrophoresis conditions: 57 cm (50 cm to the detector)×75 μm uncoated fused-silica capillary, 25 m*M* phosphate buffer (pH 2.5), constant voltage 30 kV, temperature 25°C. A sample of lyophilized tomato leaf extract redissolved in running buffer was injected by pressure for 1 s (6 nl).

content of systemin we examined a fast and efficient method like capillary electrophoresis for monitoring the isolation and purification process. Optimal conditions for CE analysis of tomato extract were found. Satisfactory separation was achieved using 25 m*M* phosphate buffer at pH 2.5 with an operating voltage of 30 kV. Under such conditions, the peptide migrates as a positively charged molecule to the cathode. Using synthetic systemin obtained by the solid-phase method [6] we could localize the natural systemin in the crude tomato leaf extract. The systemin peak with a migration time of 6.1 min was clearly visible in the complex mixture of crude extract (Fig. 1). It was well separated from other molecules. The peak was traced through all chromatographic steps (data not shown). Chromatographic fractions were analyzed by CE and analytical RP-HPLC simultaneously. The fractions were collected according to their purity based on electrophoretic mobility and hydrophobic properties of their content to reach the purity level of 80%. In the last step the required purity level was 90%. About 100 μg of systemin (weighed as acetate salt) with 90% purity was obtained (Fig. 2). The chromatographic procedure for the isolation of systemin is shown in Table 1. Isolated and synthesized systemin had the same properties. They were determined by amino-acid analysis and amino-acid sequence, CE migration time, analytical RP-HPLC retention time and molecular ion. All parameters for isolated systemin are shown in Table 2. Amino-acid sequence analysis showed the expected amino-acid composition and sequence (Fig. 3). The results obtained show that capillary electrophoresis is a fast method for monitoring the separation process of systemin. The fact that systemin is clearly visible in CE analysis of plant crude extract recommends this method to analyze the presence and behavior of peptides in

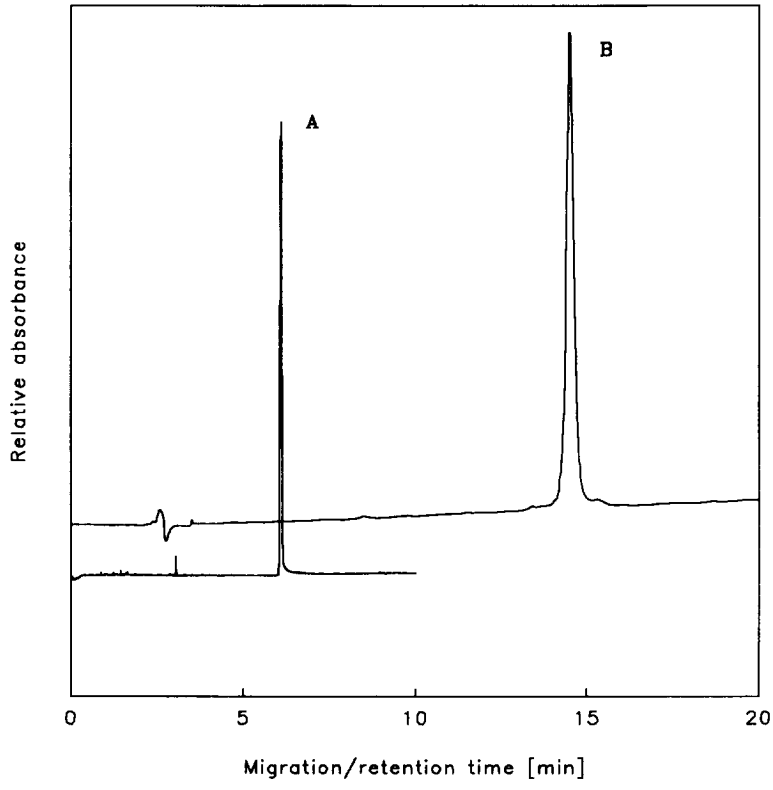

Fig. 2. (A) CE and (B) RP-HPLC analysis of pure systemin isolated from tomato leaves. (A) Conditions are the same as in Fig. 1. (B) Column: Ultrasphere ODS column (19.5×0.46 cm). Flow-rate was 1 ml/min. Solvent system: (a) 0.1% TFA, (b) acetonitrile–0.1% TFA (80:20, v/v). A linear gradient of 5–25% b for 20 min was used. Injected sample volume was 5 μl. Detection at 226 nm.

Table 1
Purification protocol for the isolation of systemin from tomato leaves

Step	Method	Mass sample loaded onto column (g)	Mass sample obtained (g)
1	Preliminary extraction	–	22.5
2	Sephadex G-25 gel chromatography	22.5	1
3	Preparative RP-HPLC	1	$2 \cdot 10^{-3}$
4	Sepharose S ion-exchange chromatography	$2 \cdot 10^{-3}$	$5 \cdot 10^{-4}$
5	Sephadex LH-20 gel chromatography	$5 \cdot 10^{-4}$	$1 \cdot 10^{-4}$

For details, see Experimental section.

Table 2
Physicochemical properties of systemin isolated from tomato leaves

Compound	Systemin
FAB-MS	
Molecular formula	$C_{85}H_{145}N_{26}O_{28}S$
Molecular mass [M+Na$^+$]	2033 (calculated); 2033.5 (found)
CE (t_M)	6.1 min
RP-HPLC (t_R)	13.8 min
Amino-acid analysis	Ala 1.00(1), Val 0.87(1), Gln 1.74(2), Ser 1.82(2), Lys 2.92(3), Pro 3.71(4), Arg 0.76(1), Asp 1.86(2), Met 0.79(1), Thr 0.84(1)

For details see Experimental section.

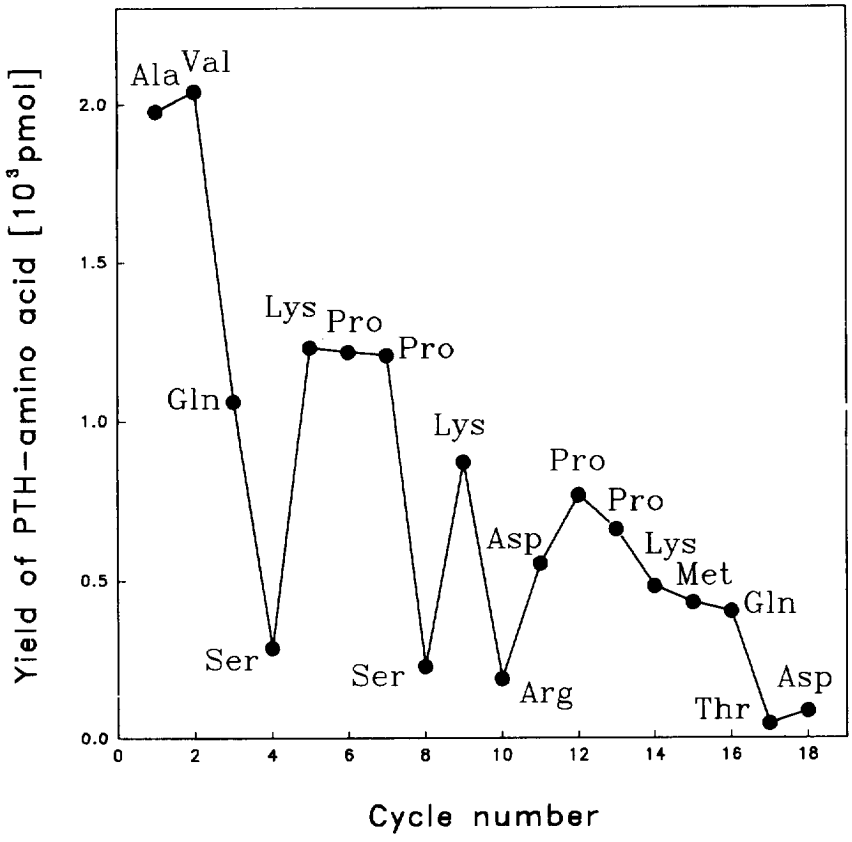

Fig. 3. Yield of PTH-amino acid at each cycle of Edman degradation of systemin isolated from tomato leaves.

plants. Systemin is the first and the only known plant polypeptide hormone. Capillary electrophoresis seems to be an efficient tool for the detection and tracing of new plant peptides, including those with other than hormonal activity.

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