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Journal of Chromatography A, 764 (1997) 331–335

JOURNAL OF CHROMATOGRAPHY A

Capillary electrophoresis of cytokinins and cytokinin ribosides[1]

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Received 16 July 1996; revised 5 September 1996; accepted 22 October 1996

Abstract

A CE separation of cytokinins and cytokinin ribosides and some other purine and pyrimidine bases has been developed. Two electrolyte systems have been tested: 150 m*M* phosphoric acid, pH 1.8 and 50 m*M* sodium dodecylsulphate+20 m*M* borate, pH 9.2. The migration times were measured and the effects of the solute structures were discussed. Preliminary experiments with plant extracts have been performed to identify the cytokinins and their ribosides. Both the systems can be used, but 150 m*M* phosphoric acid is better suited for identification of cytokinins in plant extracts, as the electropherograms are subject to fewer interferences.

Keywords: Cytokinins; Cytokinin ribosides

1. Introduction

Cytokinins are plant hormones, present in microbial, higher plant and mammalian cells in minute quantities as free bases, their ribosides and conjugates with sugars or amino acid moieties [1]. Their structure is derived from adenine or adenosine by substitution of hydrogen on the amino group in position 6 (substituent R_1). Cytokinin ribosides contain β-*o*-ribo-furanosyl group bound in position 9. The studied compounds and their structures are given in Table 1. Only *trans*-derivatives of zeatin and dihydrozeatin are present in naturally occurring plants.

Determination of cytokinins represents a considerable challenge to the analyst, as isolation and purification of cytokinins from plant material is extremely laborious and the analytes are present in trace concentrations (less than 30 ng/g). Several methods have been used for analysis of cytokinins, e.g., immunoassays (ELISA, RIA [1–6]), GC–MS [7,8] and especially HPLC [9–16] and its combinations with MS [17,18] and immunochemical methods [19,20]. Various systems have been tested in HPLC, e.g., ion-exchange [12,13] and reversed-phase (e.g., [9,11,14] chromatography.

Capillary electrophoresis has been used for the analysis of purine bases and nucleosides (e.g. [21–23], but not for cytokinins. This report describes the CZE and MEKC separations of cytokinins and cytokinin ribosides and their application to plant extracts.

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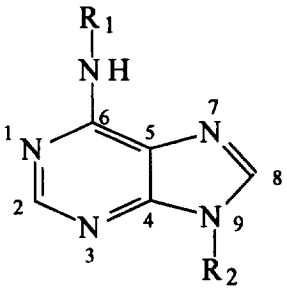
[1] Paper presented at the 10th International Symposium on Advances and Applications of Chromatography in Industry, June 30–July 4, 1996, Bratislava, Slovak Republic.

PII S0021-9673(96)00927-2

Table 1
Structures of the cytokinins studied



Compound	Abbreviation	R_1	R_2
trans-Zeatin	(Z)	$CH_2-CH_2-CH(CH_3)-CH_2OH$	
trans-Zeatin riboside	(ZR)	$CH_2-CH_2-CH(CH_3)-CH_2OH$	β-*o*-Ribofuranosyl
Dihydrozeatin	(DHZ)	$CH_2-CH=C(CH_3)-CH_2OH$	
Dihydrozeatin riboside	(DHZR)	$CH_2-CH=C(CH_3)-CH_2OH$	β-*o*-Ribofuranosyl
N^6-(Δ^2-Isopentenyl)adenine	(IPA)	$CH_2-CH=C-(CH_3)_2$	
N^6-(Δ^2-Isopentenyl)adenosine	(IPAR)	$CH_2-CH=C-(CH_3)_2$	β-*o*-Ribofuranosyl
N^6-(Benzylamino)purine	(BAP)	$NH-C_6H_5$	
N^6-(Benzylamino)purine riboside	(BAPR)	$NH-C_6H_5$	β-*o*-Ribofuranosyl

1.1. Experimental

Analytical grade chemicals were used throughout. Cytokinin derivatives were supplied by Sigma (Heidelberg, Germany). Deionized water was used to dissolve the samples.

Capillary electrophoresis was carried out on a Crystal CE, Model 310 instrument, with a variable wavelength UV photometric detector (ATI Unicam, Cambridge, UK).

Three electrolytes were tested: (A) 150 m*M* phosphoric acid, pH 1.8; (B) 20 m*M* borate+50 m*M* SDS, pH 9.3; (C) 30 m*M* phosphate+50 m*M* SDS, pH 6.0.

A capillary of 75 μm I.D., length to the detector, l_D=61 cm, total length l_T=77 cm, was used in the measurements. The conditions for pneumatic injection and the voltage applied differ in dependence on the solute concentration and the electrolyte used and are given in the figure captions. For zeatin, the theoretical plate numbers per metre were 216 000 (system A) and 110 000 (system B), respectively.

1.2. Extraction and purification of cytokinins [24]

The plant material (initial amounts of 9 g) was homogenized by pestle and mortar in liquid nitrogen. The cytokinins were then extracted with cold acetone (three times within 24 h). After centrifugation (8000 *g*, 10 min), the supernatant was evaporated to

dryness in vacuo. The residue was dissolved in lukewarm (38°C) distilled water acidified with HCl to pH 3.5. Butanol saturated with acidified water was then added to the aqueous extract and the two phases were stirred. The butanolic phase was extracted three times with acidified water and the resulting aqueous phases containing cytokinins were combined. The aqueous phase was neutralized with KOH to pH 7.0 and mixed with an equal volume of alkaline butanol (butanol:ammonia 9:1) to transfer cytokinins into the organic phase. The aqueous phase was extracted three times with alkaline butanol and the resulting organic phases were combined. The organic phase thus obtained was evaporated to dryness in vacuo. The residue was dissolved in ca. 1 ml of 50% (v/v) aqueous ethanol and passed through a Silica-Cart cartridge (Separon^{TM} SGX C_{18} 60 μm, Tessek, Prague, Czech Republic). The sample was again evaporated to dryness and dissolved in 50 μl of 50% (v/v) aqueous ethanol. This extraction and purification procedure has the following advantages over the previous ones (e.g. [19,20]): it is rapid, mild and cheap. It is solely based on the cytokinin solubility in water at varying pH and in organic solvents; the cytokinins remain unchanged, including those bound to phosphate, ribosides, etc. Other approaches cause cytokinin dissociation from natural bonds and provide only the sum of all the cytokinins, not individual types. The overall recovery was tested by adding a standard 3-hydroxy-BAP labelled with tritium to the initial plant material and the value, 85%, was calculated from the β-activity measurements before and after all the operations.

2. Results and discussion

System C has been recommended in the literature [21,23] for the separation of purine bases and nucleosides. This system was found to be unsuitable for the separation of cytokinins and cytokinin ribosides. System A has been successfully applied to the separation of bee venom components and various plant allergens [25,26]. Cytokinin bases have a basic pK_a around 4.2 and acidic pK_a around 9.8 [1]. At pH 1.8, cytokinin bases occur almost entirely in the cationic form. The neutral form predominates at a pH approximately two units above the basic pK_a and two units below acidic pK_a. Two pH units above acidic pK_a, the anionic form prevails. Cytokinin ribosides exhibit only basic pK_a. The EOF at pH 1.8 is negligible and the studied compounds are separated in dependence on their charge to mass ratios (Table 2). Therefore, cytokinins migrate faster than the corresponding cytokinin ribosides (Fig. 1A). The separation in this system is good, with the exception of Z/DHZ couple, which was only partially separated (the resolution R=0.66). The same problems were encountered in HPLC [11].

Table 2
Electrophoretic mobilities of cytokinin derivatives in systems A and B

Analyte	μ_a ($\times 10^{-4}$) A	μ_a ($\times 10^{-4}$) B
Z	4.91	4.63
DHZ	4.99	4.52
IPA	5.16	2.95
BAP	4.83	3.94
ZR	4.40	5.24
DHZR	4.27	5.29
IPAR	4.72	3.19
BAPR	4.17	4.93

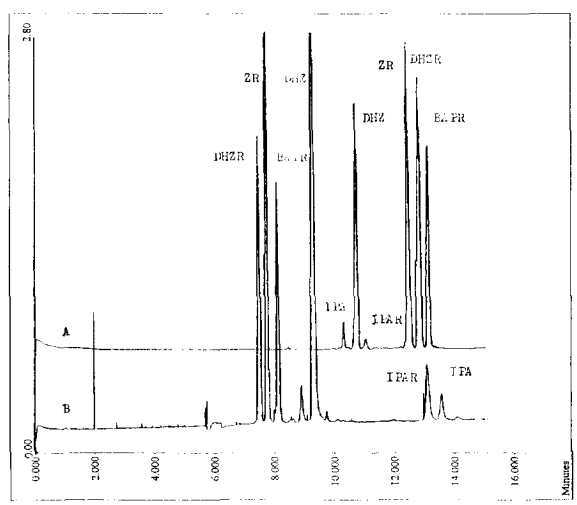


Fig. 1. Separations of IPA, DHZ, IPAR and ZR, DHZR and BAPR in systems A and B. System A: 150 mM phosphoric acid, pH 1.8, voltage 20 kV, injection 10 mbar, 0.05 min. System B: 20 mM SDS+50 mM borate, pH 9.23, voltage 20 kV, injection 10 mbar for 0.1 min. Concentration of standard solution: ca. 0.5 mg/ml except for IPA and IPAR, where the concentration was 0.1 mg/ml.

In a micellar system of the SDS-borate buffer at pH 9.3 (system B), the dissociation of cytokinins is suppressed. On the contrary to system B, cytokinin ribosides migrate faster than cytokinins in the micellar system (Table 2, Fig. 1B), due to their lower hydrophobicity in comparison with the free bases. The separation of the Z/DHZ couple is slightly better than in system A, as the resolution is $R=0.95$. The separation of DHZR/ZR is poorer in system B than in system A ($R=0.55$ in system B compared with $R=1.2$ in system A).

Cytokinins strongly absorb in the region of 220 to 300 nm, with the molar absorption coefficient of ca. 15 000. The λ_{max} depends on the position of the substituents and the pH; cytokinin bases have λ_{max} 273 nm at pH 2 and 275 nm at pH 11, 9-substituted compounds have λ_{max} 265 nm at pH 2 and 269 nm at pH 11. UV detection at 265 nm was employed in this work. Under these conditions, the limits of detection for Z and ZR (calculated from the signals equal to three times the peak-to-peak noise) were 6 and 9 μg/ml, which correspond to amounts of 36 and 45 pg in the injected volumes of 6 nl, respectively. The detection limits at 190 nm were ten times higher due to a higher noise.

The results were applied to the analysis of plant extracts. Extracts from wheat, tobacco and beet were prepared according to the procedure given in Section 2 and analyzed in both A and B electrolyte systems (Figs. 2 and 3). In beet (Fig. 2A) and tobacco (Fig. 2B) extracts, the presence of ZR and IPAR was proved by the RIA method. Fewer interferences were observed in 150 m*M* phosphoric acid at pH 1.8; neutral and negatively charged species present in the extracts do not migrate. The separations of the wheat extract in the two electrolyte systems, detected at 265 nm, are compared in Fig. 3; a CE separation of wheat extract detected at 190 nm is also shown. The concentration of cytokinins in this extract is not known; however, it follows from the comparison of the migration times that IPA might be present in the extracts. The peak with a migration time of 8 min has the same position as that for adenine. Further experiments on the identification and quantitation of cytokinins in the extracts are in progress.

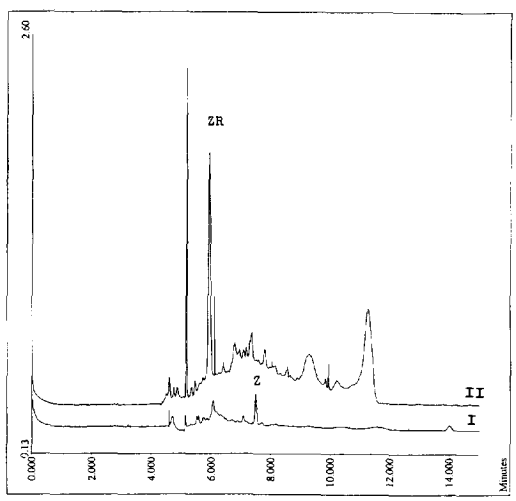


Fig. 2. Separation of extracts of beet (I) and tobacco (II) in system B. Electrolyte: 20 m*M* SDS+50 m*M* borate, pH 9.23, voltage 20 kV, injection 20 mbar for 0.1 min.

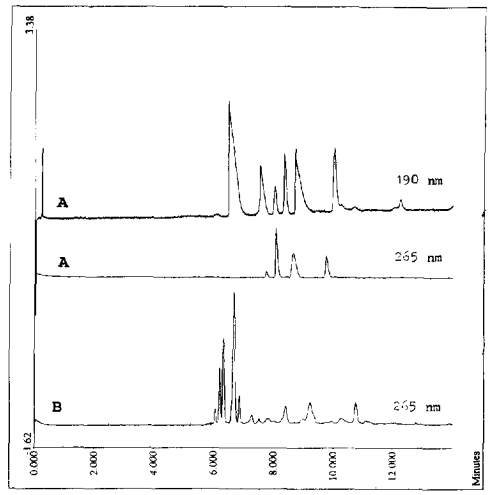


Fig. 3. Separations of wheat extract in systems A and B. System A: 150 m*M* phosphoric acid, pH 1.8, voltage 15 kV, injection 10 mbar, 0.05 min. System B: 20 m*M* SDS+50 m*M* borate, pH 9.23, voltage 20 kV, injection 20 mbar for 0.05 min.

3. Conclusions

It has been found that capillary electrophoresis is a suitable technique for analysis of cytokinins and their ribosides. Compared with the methods employed so far for these substances (GC–MS and HPLC), the present approach has the following advantages: It removes the necessity of analyte derivatization which

is required by GC–MS and uses substantially smaller samples compared with HPLC. Moreover, it is faster, cost of analysis is lower, the separation is more efficient and the method suffers from fewer interferences than HPLC–UV.

The results obtained on the plant extracts are preliminary and are being elaborated and tested. The results are indispensable in the molecular biology study of the role of cytokinins in plants.

Acknowledgments

The ATI Unicam company is thanked for kindly providing us with a demonstration CE instrument.

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