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Study of cytokinin metabolism using HPLC with radioisotope detection

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Abstract

High performance liquid chromatography (HPLC) with an on-line flow-through radioactivity detector was used to monitor the metabolism of cytokinins ([^{3}H]6-benzylaminopurine and [^{3}H]6-benzylaminopurine riboside) after their incorporation into wheat seedlings. The production and conversion of individual metabolites was assayed within a short time interval (0.5–3 h). Extraction recoveries from plant tissue proved to be 85%. The uptake of both cytokinins was very rapid and differences in their metabolism were already perceptible after 30 min. The individual metabolites were identified as adenine (Ade), adenosine (Ado), benzyladenine-9-glucoside (BA-9G), 6-benzyladenine (BA) and benzyladenosine (BAR). The method is very fast, sensitive and very useful for metabolic studies. The detection limit was 40 pg (220 Bq) for BA at the level of 2 ng ml^{-1}.

Keywords: Metabolites; High performance liquid chromatography; Detection limit

1. Introduction

Cytokinins are encountered in nature as highly active regulators of numerous physiological processes such as cell division, differentiation, plant growth and development. They function as anti-stress and anti-senescence agents and bring about enhancement of crop yield, etc. Their biological activity depends on their chemical structure and type. The level of cytokinins is controlled by other endogenous phytohormones. Cytokinins are found in plant materials in very small concentrations as free bases, their ribosides or conjugates with sugars or amino acids [1].

The importance of cytokinins and the demands for their determination gave rise to a number of methods for their analysis and detection, ranging from biotests to modern techniques such as high performance liquid chromatography (HPLC), RIA, ELISA, GC-MS and LC-MS and their combinations [2–14]. Each of the methods has its specific merits and shortcomings. Introduction of immunochemical methods brought about a considerable reduction in the amount of plant material necessary for analysis. However, unless specific antibodies are available, the plant extracts have to be separated by HPLC since crude extracts may contain interfering substances that distort the results of analyses.

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GC-MS is a highly sensitive and reliable method for identification and quantitation but the necessary derivatization may bring problems. The use of capillary electrophoresis for the analysis of some cytokinins has recently been described by Pacáková [15]. One of the state-of-the-art methods is a combination of immunochemical techniques with atmospheric-pressure chemical-ionization mass spectrometry (LC/APCI-MS) [13].

Metabolic studies of some cytokinins have been performed by using compounds labelled with radioactive isotopes, mostly tritium or ^{14}C [16–18]. The radioactivity detection is then done by liquid scintillation counting of a large number of fractions of the chromatographic eluate.

Our study used HPLC with an on-line flow-through radioactivity detector for monitoring the metabolites of tritiated benzyladenine and benzyladenosine after their incorporation into wheat plants.

2. Experimental

2.1. Chemicals and radiochemicals

Standards of the cytokinins 6-benzylaminopurine (BA), 6-benzylaminopurine riboside (9R-BA), Ade, Ado, 3-hydroxybenzyladenin (3-OH-BA) and 9G-BA were purchased from Sigma-Aldrich (Prague, Czech Republic). ^{3}H-standards of the same cytokinins were synthesized by Dr J. Hanuš in the Isotope Laboratory. The specific activity of these radioactive standards was ≈ 1 TBq mmol^{-1} except for 9G-BA which had a minimum specific activity of 0.26 TBq mmol^{-1}. The radiochemical purity of all compounds was higher than 98%. All cytokinin standards have been labelled with tritium at position 2 of the purine ring. The labelling at this position is stable. The tritiation synthesis was performed by alkylation of 2-[^{3}H]adenosine. The only exception was BA-9G which was prepared by catalyzed exchange reaction. The solvents, methanol and acetonitrile of gradient grade for HPLC analysis, were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade, purchased from Lachema (Prague, Czech Republic).

2.2. Plant material

The plant tissue under study was wheat shoots (*Triticum aestivum* var. *Jara*). Six-day old wheat seedlings were used which had been grown in distilled water at 21°C.

2.3. Incorporation of [^{3}H]BA and [^{3}H]BAR

Wheat shoots were placed into 5 ml water with [^{3}H]BA or [^{3}H]BAR (13.6 μCi ml^{-1}), weighed and frozen. The cytokinins were extracted and purified by the method described in Section 2.4 and eventually separated by HPLC.

2.4. Extraction and purification of cytokinins

The plant material (initial amounts of 9 g) was homogenized by pestle and mortar in liquid nitrogen. Cytokinins were then extracted with cold aceton (three times within 24 h). After centrifugation ($8000 \times g$, 10 min) the supernatant was evaporated in vacuo to dryness. The residue was dissolved in lukewarm (38°C) distilled water acidified to pH 3.5 with HCl. A triple extraction of an aqueous solution of cytokinins with butanol saturated with water acidified to pH 3.5 removed chlorophyll and other impurities, along with traces of cytokinins. These traces of cytokinins were then recovered by separating the butanol layer and back-extracting it three times with water acidified to the same pH. The acidified aqueous layers containing cytokinins were combined and then neutralized to pH 7 with KOH. A subsequent multiple extraction of this aqueous layer with alkaline butanol (butanol/ammonia 9:1) served to transfer the cytokinins into the butanol phase and free them of salts and other admixtures, which remained in the aqueous layer.

The final organic phase was evaporated in vacuo to dryness, the residue dissolved in 3 ml of 50% (v/v) ethanol and passed through a Silica-Cart cartridge (Separon™ SGX C 18 60 μm, Tessek, Prague, Czech Republic). Then this cartridge was washed twice with 1 ml of 50% ethanol and all effluents combined. The resulting volume of 5 ml was evaporated again to dryness and the residue dissolved in final volume of 1 ml 50% ethanol.

Sample volumes of 20 or 50 μl were injected onto HPLC system.

2.5. Extraction efficiency

The efficiency of this extraction method was determined using a known amount of a radioactive inner standard of [^{3}H]3-OH-BA. The starting and final values of radioactivity were checked on a liquid scintillation counter (BECKMAN LS 6500). The calculated efficiency was 85% (coefficient of variation (CV) ranged from 6 to 9.5%, $n = 5$)

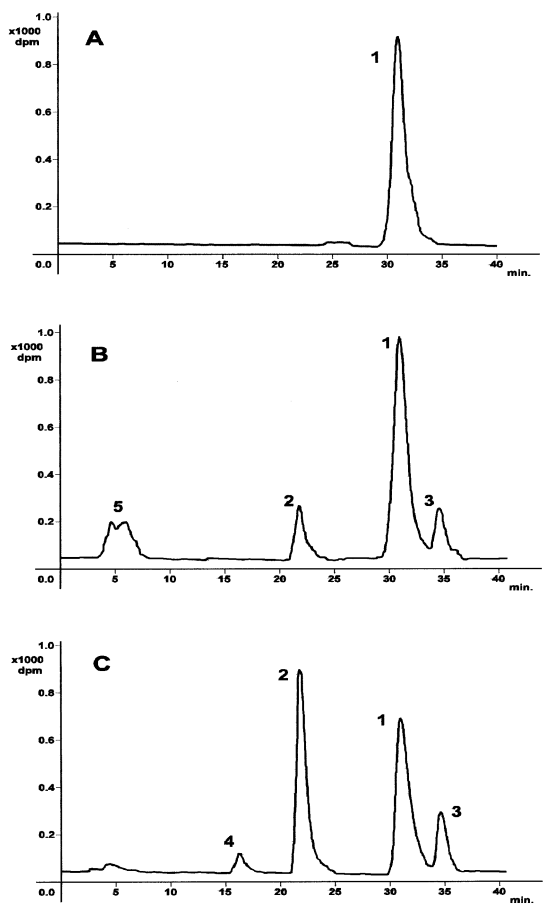

Fig. 1. A typical radiochromatogram of tritiated benzyladenine metabolites observed at 0 h (A), 0.5 h (B) and 3 h (C) after the beginning of benzyladenine incorporation into wheat seedlings. Peaks: 1, [^{3}H]BA; 2, [^{3}H]BA-9G; 3, [^{3}H]BAR; 4, unknown; 5, [^{3}H]Ade, Ado region.

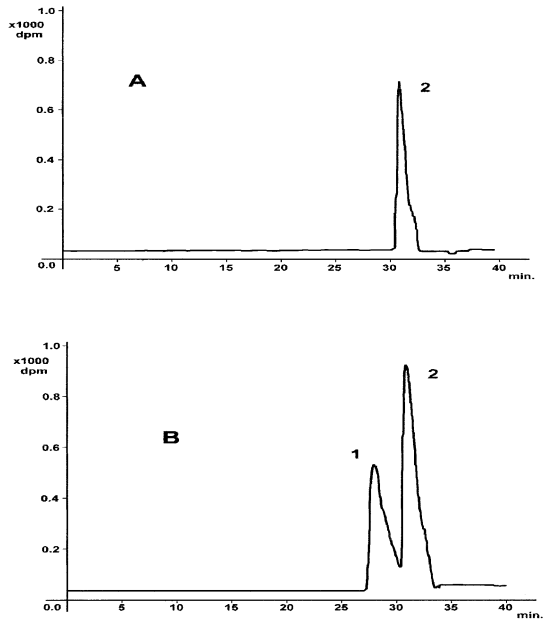

Fig. 2. A typical chromatogram of a tritiated benzyladenosine metabolite determinated at 0 h (A) and 1 h (B) after the beginning of benzyladenosine incorporation. Peaks: 1, [^{3}H]BAR; 2, [^{3}H]BA.

2.6. Instrumentation and chromatographic conditions

HPLC measurements were performed using a Waters liquid chromatograph (Waters, Milford, MA) with an on-line radioisotope flow-through detection system from Beckman (Beckman Instruments, Irvine, CA).

The HPLC system consisted of a Model 600 solvent delivery system and a Model 490E programmable multiwavelength UV detector. A Model 7125 injector (Rheodyne, Cotati, CA) was used for sampling. Model 171 Beckman radioisotope detection system was used with a solvent delivery module for scintillator pumping. Radioactivity was measured by the admixture method. After passing through the UV detector the column eluate was mixed continuously with the liquid scintillator cocktail READY FLOW III (Beckman) in a ratio of 1:2.5 in an on-line connected high efficiency mixer (Beckman). The resulting mixture was passed through a 1-ml

scintillation cell. The cell was positioned between the cathodes of two photomultipliers operating in coincidence to reduce the background. The threshold was set at 0.03% and the residence time was 0.417 min.

All HPLC analyses were performed on a stainless-steel analytical column (150 × 4 mm I.D.) LiChroCART (Merck), packed with 5 μm LiChrospher 100 RP-18 endcapped, protected by a guard column (4 × 4 mm I.D.) packed with the same packing (Merck).

Three solvents were used as the mobile phase; (A) methanol with acetonitrile (1:1); (B) 10% solvent A with 40 mM acetic acid buffered to pH 3.5 with triethylamine; and (C) 80% solvent A with the same acetic acid. A linear gradient programme was used. The ratio of the solvents changed (B, 90:75%), (C, 10:25%) over a 20-min period. The applied flow rate was 0.8 ml min^{-1} under continuous degassing with helium. The wavelength of the UV detector was set at 230 nm, 0.05 AUFS.

Two simultaneous detector reports, UV absorbance and radioactivity, were obtained for each chromatogram. The radioactivity data were obtained as CPM versus time.

2.7. Precision

The precision was calculated from data obtained during a 10-h period and was expressed as the CV for five measurements. The resulting values ranged from 0.5 to 7.6%.

3. Results and discussion

Radioisotope detection was used to monitor the metabolites of tritiated cytokinins BA and BAR formed within a short time interval, 0.5–3 h, after their incorporation into the wheat seedlings.

After prepurification by solid-phase extraction, samples of plant material extracts were analyzed before and after the incorporation of ^{3}H-labelled BA and BAR. The analyses used, simultaneously, a UV and radioactivity detection. Whereas the UV detector showed no differences, the radiochromatograms showed the presence of several metabolites.

Prior to the analysis of plant extracts, a mixture of tritiated cytokinin standards was monitored under the same conditions. These included above all aromatic cytokinins with a benzene ring substituted in position N^6 of adenine. The mixture of standards contained BA, BAR, 3-OH BA, BA-9G, Ade and Ado. All these substances exhibited a base-line separation with the exception of 3-OH BA and BA-9G, which were poorly separated.

The total uptake of ^{3}H-labelled BA and BAR and the production of their metabolites were followed at short intervals, viz. 0.5, 1, 2 and 3 h after the beginning of incorporation. Both cytokinins were incorporated into the plant tissue very rapidly but the number and nature of the detected metabolites differed.

The tritiated benzyladenine was metabolized to several compounds after a mere 30 min while tritiated benzyladenosine remained unchanged. The BA metabolites were adenine and adenosine with retention times of 2.8 and 5.1 min, a metabolite corresponding to BA-9G in its retention time of 21.7 min and another metabolite whose retention time corresponded to BAR. After 1 h, we observed only a slight quantitative increase but no qualitative change in the products.

More sizeable changes were observed after 3 h of incubation when the adenine and adenosine metabolites disappeared while an unknown compound with a retention time of 16 min appeared.

The metabolic degradation of [^{3}H]BAR during the first 30 min was zero but the next 30 min witnessed a considerable increase in a single metabolite which corresponded in its t_R to the BA free base in a relative concentration of 40%.

The radiochromatograms illustrating the metabolism of benzyladenine and benzyladenosine are given in Figs. 1 and 2 and the time evolution of the relative proportion of all substances is documented in Table 1. The identification of the arising compounds was performed by the method of internal standard and by comparing the retention times with standards. The chemical structure of these compounds will have to be confirmed by mass spectrometry (paper in preparation).

The detection of tritium-labelled compounds can be done either in a cell with solid scintillator

Table 1
Retention time (t_R) and relative concentration (c_{rel}) of [^{3}H] labelled cytokinin metabolites in analysed samples after [^{3}H]BA and [^{3}H]BAR incorporation

[^{3}H] labelled cytokinin metabolites	t_R^a (min)	[^{3}H]BA incorporation				[^{3}H]BAR incorporation			
		Incorporation period (h)				Incorporation period (h)			
		0	0.5	1.0	3.0	0	0.5	1.0	3.0
		Relative concentration[b] c_{rel} (%)				Relative concentration[b] c_{rel} (%)			
BA	30.9	100	68.1	64	38	n.d	n.d	38.9	74.2
Ade, Ado	2.8, 5.1	n.d.	14.2	14.8	<1	n.d.	n.d.	n.d.	11.4
BA-9G	21.7	n.d.	9.3	11.8	43.1	n.d.	n.d.	n.d.	3.4
BAR	34.6	n.d.	7.5	9.1	8.6	100	n.d.	61.5	11
Unknown	15.9	n.d.	n.d.	n.d.	7.8	n.d.	n.d.	n.d.	n.d.

n.d., not detected.
[a] Data are given as mean values.
[b] Precision of determination (CV), $n = 5$ ranges from 0.5 to 7.6%.

or using a liquid system, i.e. flow-through cell with a liquid scintillation cocktail. In this study we used the flow-through cell in order to prevent residual sorption and increase in the background on a solid scintillator. These phenomena have therefore been eliminated. The sensitivity of radiodetection depends on the value of the residence time in the measuring cell, detector efficiency and the specific activity of the radioactive compound. The absolute detection limit in our system, defined by a signal-to-noise ratio of 3, was in the range of 100–260 Bq for cytokinin standards (concentration of 2 ng ml^{-1}).The peaks on the radiochromatograms were symmetrical without any signs of tailing. The method was found to be completely reproducible with CV (%) values ranging from 0.5 to 7.6% over the period of 10 h.

The calibration curves of standard solutions of known amounts of cytokinin standards were linear in the range 100–10 000 Bq ($r = 0.996$).

In contrast to the response of the UV detector, the response of the radioactivity detector is fully independent of the chemical structure of the analyzed compounds. This is especially advantageous in analyses of trace amounts of cytokinins in plant tissue extracts containing considerable amounts of substances absorbing in the UV region. The method should not serve for absolute determination but is suitable for rapid assays of relative concentrations of the arising cytokinin metabolites and monitoring of the time course of their biosynthesis.

The method enabled us to compare the metabolic conversions of benzyladenine and benzyladenosine in wheat shoots at the same time intervals during a very short time period, viz. 0.5–3 h, under the same cultivation and incubation conditions.

The growth and division of plant cells was confirmed to depend significantly on the base. For this reason, BAR after incorporation is first metabolized to BA which is further metabolized in the same way as when incorporated alone. This involves degradation, after 30 min, to adenine–adenosine, the amounts of which depend on the level of endogenous cytokinins. The degradation then proceeds to BAR in the transport form and to reserve 9G-BA.

These results are not at variance with previous data on BA and BAR metabolism in various plants published in our and other laboratories.

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