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Note

Thin-layer chromatography of cytokinins on a mixed layer of polyvinylpyrrolidone and calcium sulphate*

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The determination of cytokinins in plant extracts by physico-chemical methods necessitates a long, rigorous purification procedure to remove the numerous water-soluble plant components which are likely to interfere in the analysis. In current investigations on the cytokinin complex occurring in stored cabbage heads[1], a new high-pressure liquid chromatography system has been examined and since an attempt was made to use UV absorption measurements as an indication of cytokinin activity, extracts of reasonable purity were essential.

Many different methods of preliminary purification of cytokinin-containing plant extracts have been used but some of these techniques, particularly the use of ion-exchange chromatography where solutions of high ionic strength are employed, are questionable because of the possibility of degradation of naturally occurring compounds during the separation process. The use of insoluble polyvinylpyrrolidone (PVP) on liquid chromatographic columns as a method for separating cytokinins has been described previously[2] and this system has the advantage that only weak buffers are used as eluants, and recovery rates are high.

PVP has also been used successfully on thin-layer chromatography (TLC) plates in a mixture with cellulose for the separation of anthocyanins[3] and was shown to have the advantage over other layers in that very compact spots were obtained. The use of 100% PVP plates[4] was not recommended since an irregular solvent front was obtained and separations were incomplete.

In this communication, the further use of PVP in a mixture with $CaSO_4 \cdot 2H_2O$ on TLC plates for the purification and separation of cytokinins in plant extracts is described. It is envisaged that TLC on such plates could replace the questionable use of ion-exchange chromatography for the preliminary isolation of cytokinins and could also be used as a method for separating free base cytokinins from the related ribosides.

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EXPERIMENTAL

Preparation of plates

Either ungraded insoluble polyvinylpyrrolidone (Calbiochem, Los Angeles Calif., U.S.A.) or Polyclar AT (General Aniline and Film Corp., New York, N.Y., U.S.A.) was ground with AnalaR $CaSO_4 \cdot 2H_2O$ in a mortar in the proportions 1:1 (v/v) or 40:100 (w/w), respectively. Plates of approximately 0.4 mm thickness were prepared by spreading a homogenate of 25 g of this mixture and 55 ml of distilled water on 20 × 20 cm glass plates. The coated plates were allowed to set at room temperature.

Preparation of chemical standards and plant extracts

Cytokinins, purines and their related ribosides, and nucleic acid pyrimidines were obtained from various chemical companies and 100 mg/l ethanolic solutions were prepared for spot loading on to the TLC plates. The sample size was 5 μl.

Heads of Danish cabbage cv. Green Winter were extracted to yield crude *n*-butanol-soluble fractions containing cytokinin activity[5] and these were used to assess the efficiency of the TLC systems in isolating cytokinins from the other plant components and in separating the free bases from the ribosides.

Detection of activity on the TLC plates

The pure standards after chromatography were detected as blue spots by spraying the dry plates with a bromphenol blue/silver nitrate reagent for purines. The quantity of the chemical which could be recovered from the plate was determined by scraping off the relevant zone, eluting the PVP/$CaSO_4$ twice with 70% methanol and injecting the reduced methanolic extract into a high-pressure liquid chromatograph. The initial and final concentrations of the standards were measured by UV absorption and recovery rates were determined.

Biological activity of the cytokinins in cabbage extracts was determined by similarly scraping off the PVP/$CaSO_4$, extracting with 70% methanol and measuring the activity in the eluate from each R_F value with the modified *Amaranthus* betacyanin bioassay[6]. Other components of the cabbage extracts were apparent as pigmented zones on the plates or as fluorescent bands when the plates were viewed under UV light.

Chromatography

After a number of preliminary experiments two solvent systems were selected to develop the plates.

Solvent A. A mixture of 0.013 *M* phosphate buffer pH 6.4 with 25% acetone was used to move all the cytokinins to the centre of the plate and to separate them from free pyrimidine bases and other UV absorbing phytochemicals.

Solvent B. A mixture of 0.03 *M* borate buffer pH 8.8 with 10% acetone was used to separate the free bases from their related ribosides. The use of borate solutions to separate sugars and ribosides from free bases is well-documented and similar systems have been used as standard techniques for the paper chromatography of cytokinins.

The plates were developed in glass tanks at room temperature and were then air dried prior to spraying or bioassay.

RESULTS AND DISCUSSION

The main purpose of this investigation was to develop a TLC system to replace the use of ion-exchange chromatography. We wished to isolate the cytokinins occurring in plant tissues and separate them from other UV absorbing phytochemicals, particularly the nitrogen bases of nucleic acids. Satisfactory separation was achieved by using solvent A except for the fact that adenine and adenosine could not be removed by this means (Table I). Unfortunately, both these bases are likely to occur in the cytokinin fraction obtained from plant tissues and interfere with the determination by UV absorption of cytokinins separated on a high-pressure liquid chromatogram. Thus, some other separation procedure would be required following TLC unless cytokinin activity is assessed by a bioassay in which adenine and adenosine show no biological activity.

TABLE I

R_F VALUES OF CYTOKININS AND NITROGEN BASES SEPARATED BY CHROMATOGRAPHY ON PVP/$CaSO_4$ THIN LAYERS

Solvent systems: (A) 0.013 *M* phosphate pH 6.4 + 25% acetone; (B) 0.03 *M* borate pH 8.8 + 10% acetone.

Classification	*Compound*	*Solvent A*	*Solvent B*
Free-base cytokinins	Zeatin (Z)	0.52	0.23
	N^6-(Δ^2-Isopentenyl)-aminopurine (2iPA)	0.40	0.16
	N^6-Furfurylaminopurine (Kinetin) (K)	0.32	0.12
	N^6-Benzylaminopurine (BA)	0.43	0.17
	Dihydrozeatin (DHZ)	0.55	0.24
Riboside cytokinins	Zeatin riboside (ZR)	0.60	0.78
	2iPA riboside (2iPAR)	0.51	0.64
	Kinetin riboside (KR)	0.45	0.60
	BA riboside (BAR)	0.34	0.46
Purines	Adenine (Ad)	0.52	0.34
	Guanine (Gua)	0.20	0.36
	N^6,N^6-Dimethylaminopurine (DMA)	0.56	0.32
	Adenosine (AR)	0.63	0.98
	DMA riboside (DMAR)	0.60	0.98
Pyrimidines	Cytosine (Cy)	0.81	0.80
	Uracil (Ura)	0.77	0.80
	Thymine (Thy)	0.76	0.80

All the known cytokinins are N^6-substituted adenines and Fig. 1 shows that these can be separated from most plant pigments and many interfering compounds occurring in cabbage extracts by TLC in solvent A. Thus, considerable purification of cytokinin-containing extracts is possible by TLC on PVP/$CaSO_4$ plates and elution of the zone from R_F 0.25 to 0.75 with 70% methanol or ethanol. With cabbage extracts a convenient marker of intense blue fluorescence appeared just in front of the cytokinin zone on exposing the plate to UV light so that the coating between this zone

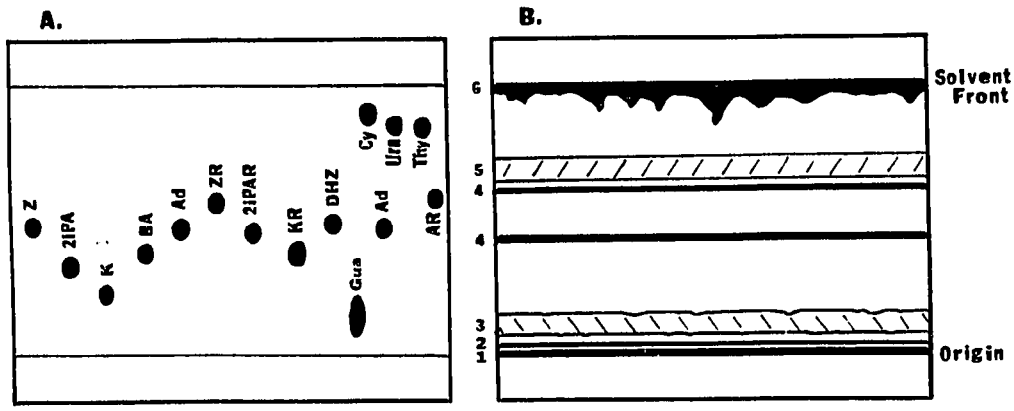

Fig. 1. Thin-layer chromatograms run in solvent A of (A) standard chemicals and (B) an *n*-butanol extract of 10 g of cabbage head tissue. (A) For code to standards see table I. (B) 1 = Yellow pigmented zone at loading line; 2 = purple UV fluorescent zone; 3 = intense-blue UV fluorescent zone; 4 = purple UV fluorescent zones; 5 = pink pigmented zone. 6 = gums and green-yellow pigments.

and R_F 0.7 was removed and further purified and bioassayed in our cytokinin studies.

The PVP/$CaSO_4$ system can also be used to separate free-base cytokinins from their related ribosides (Fig. 2) when Solvent B is used to develop the plates. In our studies, a satisfactory separation was obtained by dividing the plate at R_F 0.4 following development so that R_F 0.0 to 0.4 contained the free bases whilst the ribosides ran between R_F 0.4 to 1.0.

In our recovery studies up to 90% recovery of both zeatin and zeatin riboside was achieved and we were able to determine the cytokinin activity of cabbage extracts by bioassay of the 70% methanol eluates from plates run in both solvents (Fig. 3). Chromatography in solvent B indicated that both free bases and ribosides were present in the extract.

The resolution of marker spots in both solvents was good with very little streaking and solvent fronts were regular, these results being similar to those described for the separation of anthocyanins on PVP-cellulose plates[3]. When 100% PVP was used

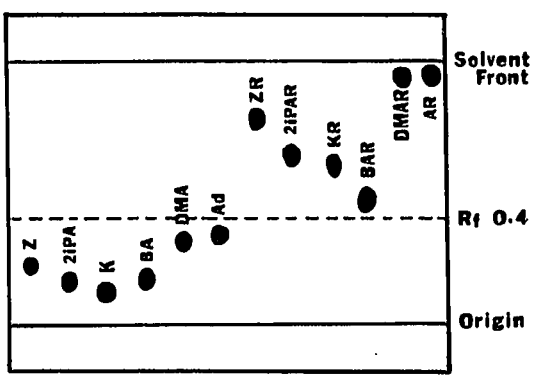

Fig. 2. Thin-layer chromatograms run in Solvent B of cytokinins and purines and their related ribosides. For code see table I.

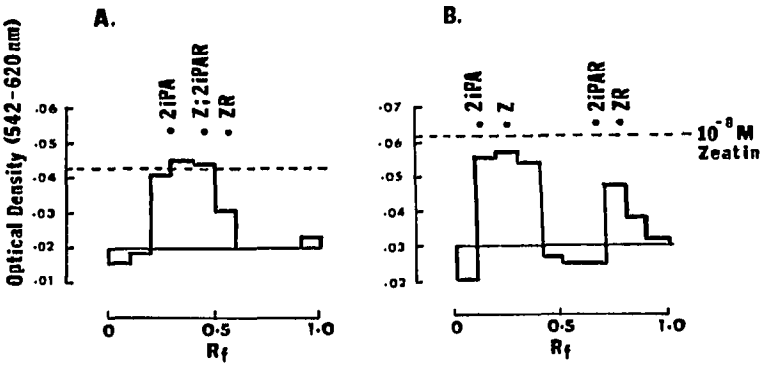

Fig. 3. *Amaranthus* betacyanin bioassay of 100 g cabbage extracts run on TLC plates in (A) solvent A and (B) solvent B.

or if the amount of $CaSO_4$ was reduced, the coating cracked badly and resolution was poor. The incorporation of $CaSO_4$ into the coating ensured a very stable layer with good separatory properties.

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