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TRACE ENRICHMENT OF CYTOKININS USING NEUTRAL POLYSTYRENE RESINS*

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SUMMARY

Two neutral polystyrene resins, Amberlite XAD-2 and Porapak Q, have been tested for effectiveness in the quantitative recovery and trace enrichment of cytokinins from aqueous solutions. Both resins retained all of the cytokinin-active bases and ribonucleosides tested. Elution with 70% ethanol permitted quantitative recovery of cytokinins from the resins. Under the test conditions used here, adenine and the major ribonucleosides were either incompletely retained or not retained at all by the two resins. Quantitative recovery of cytokinin activity from hydrolysates of tRNA and from cultures of *Agrobacterium tumefaciens* was demonstrated using Amberlite XAD-2.

INTRODUCTION

The isolation of cytokinins from natural sources has been facilitated by the introduction of procedures involving trace enrichment on reversed-phase C_{18}-silica columns[1,2]. Recently, Stafford *et al.*[3] have utilized the neutral polystyrene resin, Amberlite XAD-2, in the isolation of the cytokinin-active constituents of coconut milk. The binding and recovery from Amberlite XAD-2 of a number of cytokinins bearing N^6-isoprenoid side-chains were also demonstrated. To test further the utility of neutral polystyrene resins in the analysis of cytokinins, we have examined the effectiveness of two such resins, Amberlite XAD-2 and Porapak Q, in the quantitative recovery and trace enrichment of cytokinins from a number of sources.

Amberlite XAD-2 and Porapak Q have both found a number of analytical applications. Amberlite XAD-2 and the closely related XAD-4 have been used in the removal of phenolic compounds and other secondary metabolites from plant enzymes preparations[4,5], in the analysis of trace organic constituents in a variety of aqueous systems[6,7], and in chromatographic separations of various classes of organic compounds[8]. Porapak Q is marketed as a support for gas chromatography. It has been

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used in liquid chromatography for the fractionation and desalting of amino acids and peptides[9], for the separation of steroids[10], and indole derivatives[11], and for some chromatographic separations involving cytokinins[12–14].

In the present study, the utility of Amberlite XAD-2 and Porapak Q in the quantitative recovery of cytokinins from aqueous solutions has been tested using both synthetic and naturally occurring cytokinins. The retention of adenine and the major ribonucleosides by the two resins has also been examined. The recovery of cytokinins from natural sources has been assessed using hydrolysates of transfer RNA (tRNA) and cell-free supernatants from cultures of *Agrobacterium tumefaciens*.

EXPERIMENTAL

Chemicals

Amberlite XAD-2 (20–50 mesh), Sephadex LH-20, lyophilized snake venom (*Crotalus adamanteus*), alkaline phosphatase (calf intestinal mucosa, type VII), ribonuclease T1 (*Aspergillus oryzae*), cytokinin standards, and the various purine and pyrimidine derivatives used in this study were obtained from Sigma. N^6-(Δ^2-Isopentenyl)adenosine-5′-monophosphate was obtained from Pharmacia P-L Biochemicals. N^6-Isopentyladenine was synthesized as described by Leonard *et al.*[15]. Porapak Q (100–120 mesh) was purchased from Waters Assoc. *Escherichia coli* tRNA and brewer's yeast tRNA were purchased from Boehringer-Mannheim. Wheat germ tRNA was prepared in this laboratory as described by Burrows *et al.*[16].

Biological materials

Agrobacterium tumefaciens C-58 was cultured in a defined medium as described by Kaiss-Chapman and Morris[17]. The bacterial cultures were grown at 26°C in Fernbach flasks on a rotary shaker at 150 cycles/min. The cultures were inoculated with late log phase cells (5 ml inoculum per liter medium). After a 24-h growth period, the cells were separated from the medium by centrifugation (14,500 *g*, 25 min). The cell-free medium obtained in this manner was used in studies on cytokinin isolation.

Amberlite and Porapak chromatography

Amberlite XAD-2 was hydrated in distilled water and washed successively with 70% ethanol and 95% ethanol until absorbance at 260 nm could no longer be detected in the wash solutions. The ethanol was removed completely by washing with distilled water. The washed resin was suspended in distilled water and packed into appropriate chromatographic columns. Porapak Q was prepared in a similar manner.

Chromatographic samples were applied to Amberlite or Porapak columns as aqueous solutions adjusted to pH 5.8. Following sample application, the columns were washed with ten bed volumes (Amberlite resins) or 20 bed volumes (Porapak Q) of distilled water followed by ten bed volumes of 70% ethanol. The first five and second five bed volumes of the 70% ethanol eluate were collected separately. The column eluates were taken to dryness under reduced pressure at 40°C and redissolved in the solutions appropriate to further processing or analysis.

During elution of the columns, the transition from water to ethanol frequently resulted in some mechanical disruption (channeling) of the resin bed. This did not appear to interfere with the recovery of cytokinins from the columns. Channeling

could be minimized by degassing all solvents prior to use and by using cold 70% ethanol for elution.

Sephadex LH-20 chromatography

Fractionations of cytokinins by Sephadex LH-20 chromatography were performed as described by Armstrong *et al.*[18]. Details of chromatography are given in the legend to the figure.

Hydrolysis of tRNA preparations

tRNA preparations (600 A_{260} units each) were hydrolyzed to ribonucleosides by treatment with ribonuclease T1 followed by snake venom phosphodiesterase and alkaline phosphatase[19]. The hydrolysates were adjusted to pH 5.8 and heated at 60°C for 10 min to terminate the reaction. Precipitated protein was removed by centrifugation (20,000 *g*, 10 min).

Determination of cytokinin activity

Cytokinin activity was determined in the tobacco callus bioassay[20]. All bioassay samples were incorporated into 100 ml of RM-1964 medium[20] and tested in five-fold serial dilutions as described by Armstrong *et al.*[21]. Cytokinin activity is expressed as microgram kinetin equivalents (μg KE), defined as the micrograms of kinetin (N^6-furfuryladenine) required to give the same growth response as the test sample under the specified bioassay conditions.

All bioassay samples derived from tRNA hydrolysates were acid hydrolyzed in 5 ml of 0.1 *N* hydrochloric acid (100°C, 45 min) prior to bioassay for cytokinin activity. The acid hydrolysates were neutralized (sodium hydroxide) before incorporation into the bioassay medium. Ribonucleoside fractions recovered from the Amberlite XAD-2 columns were taken to dryness *in vacuo* at 40°C prior to acid hydrolysis. Bioassay samples taken from the crude tRNA hydrolysates and from the first five bed volumes of the 70% ethanol eluate from the Amberlite XAD-2 columns were equivalent to 60 and 90 A_{260} units, respectively, of the original tRNA preparations. All other fractions were bioassayed *in toto*.

All bioassay samples derived from *A. tumefaciens* culture medium were taken to dryness *in vacuo* at 40°C and redissolved in 20 ml of distilled water by boiling in a water-bath for 45 min. The resulting aqueous solutions were incorporated into the bioassay medium. The bioassay sample taken from the unfractionated culture medium and from the first five bed volumes of the 70% ethanol eluate from the Amberlite XAD-2 column were each equivalent to 300 ml of original culture medium. The bioassay sample taken from the material that flowed through the Amberlite XAD-2 column during sample application was equivalent to 1 l of original culture medium. All other fractions were bioassayed *in toto*.

RESULTS

Recovery of cytokinin standards from aqueous solutions

Amberlite XAD-2 and Porapak Q were initially tested for effectiveness in adsorbing cytokinins from standard aqueous solutions. The ability of each resin to bind adenine and the major ribonucleosides was also examined. Aqueous solutions of the

test compounds were applied to chromatographic columns packed with the appropriate resin. Following sample application, the columns were washed extensively with distilled water. Materials that remained bound to the column under these conditions were recovered by eluting the columns with 70% ethanol. The results of these model experiments are summarized in Tables I and II.

All of the cytokinin bases and ribonucleosides tested, including both synthetic (b^6Ade, b^6Ado, f^6Ade, f^6Ado, hi^6Ade) and naturally occurring compounds (i^6Ade, i^6Ado, io^6Ade, io^6Ado), were quantitatively retained by the resins and were not removed by extensive washing with water. Adenine and the major ribonucleosides were more weakly bound and were eluted from the columns to varying degrees by the water wash. The cytokinins were quantitatively recovered from the columns by elution with 70% ethanol. The recovery of cytokinins was 90% or more complete in the first five bed volumes of the ethanol elute, and quantitative recovery was achieved by elution with ten bed volumes of 70% ethanol.

In similar tests with a neutral alkylacrylate resin, Amberlite XAD-7 (data not shown), adenine and the free base forms of the cytokinins were rather strongly bound to the resin. As a result, better separation of cytokinins from adenine and adenosine as well as more efficient recovery of the cytokinin standards was obtained with the neutral polystyrene resins than with XAD-7.

The nucleotide AMP was not retained by Porapak Q, and only a small fraction of a sample of i^6AMP bound to the resin. Both nucleotides, however, were bound by Amberlite XAD-2. In the case of i^6AMP, more than 90% of the sample was retained after elution with 70% ethanol. The binding of AMP and i^6AMP by XAD-2

TABLE I

PERCENTAGE RECOVERY OF CYTOKININS AND RIBONUCLEOSIDES FROM AMBERLITE XAD-2 COLUMNS

For each test compound, 100 ml of an 0.02 m*M* solution were applied to an Amberlite XAD-2 column (13 g, 30 × 0.9 cm I.D.). Recovery was determined spectrophotometrically by measurements at appropriate wavelengths.

*Compound**	*Flow-through (5 bed volumes)*	*Water-wash (10 bed volumes)*	*70% Ethanol eluate (bed volumes 1–5)*	*70% Ethanol eluate (bed volumes 6–10)*	*Total recovery (%)*
Ade	80	18	3	0	101
Ado	37	16	44	3	100
AMP	0	14	10	1	25
b^6Ade	1	0	88	8	97
b^6Ado	1	1	93	7	102
Cyd	90	10	2	1	103
Guo	62	23	14	1	100
i^6Ade	0	0	92	8	100
i^6Ado	0	0	90	7	97
i^6AMP	0	1	5	0	6
io^6Ade	1	1	100	1	103
io^6Ado	1	1	98	2	102
Urd	82	14	3	1	100

* Abbreviations: Ade, adenine; Ado, adenosine; AMP, adenosine-5′-monophosphate; b^6Ade, N^6-benzyladenine; b^6Ado, N^6-benzyladenosine; Cyd, cytidine; Guo, guanosine; i^6Ade, N^6-(Δ^2-isopentenyl)adenine; i^6Ado, N^6-(Δ^2-isopentenyl)adenosine; i^6AMP, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; io^6Ade, zeatin; io^6Ado, ribosylzeatin; Urd, uridine.

TABLE II

PERCENTAGE RECOVERY OF CYTOKININS AND RIBONUCLEOSIDES FROM PORAPAK Q COLUMNS

For each test compound, 100 ml of an 0.02 m*M* solution were applied to a Porapak Q column (0.33 g, 5.8 × 0.48 cm I.D.). Recovery was determined spectrophotometrically by measurements at appropriate wavelengths.

*Compound**	*Flow-through (100 bed volumes)*	*Water-wash (20 bed volumes)*	*70% Ethanol eluate (1–5 bed volumes)*	*70% Ethanol eluate (6–10 bed volumes)*	*Total recovery (%)*
Ade	90	8	1	1	100
Ado	61	18	21	1	101
AMP	98	2	0	0	100
b^6Ade	0	0	79	19	98
b^6Ado	0	0	92	8	100
Cyd	94	4	1	1	100
f^6Ade	0	0	95	4	99
f^6Ado	0	0	97	5	102
Guo	92	9	1	0	102
hi^6Ade	0	0	86	14	100
i^6Ade	0	0	100	1	101
i^6Ado	0	0	92	6	98
i^6AMP	74	10	16	1	101
io^6Ade	0	0	98	1	99
io^6Ado	0	0	98	1	99
Urd	98	4	1	1	104

* Abbreviations are as in Table I with the following additions: f^6Ade, N^6-furfuryladenine; f^6Ado, N^6-furfuryladenosine; hi^6Ade, N^6-isopentyladenine.

TABLE III

RECOVERY OF CYTOKININ-ACTIVE RIBONUCLEOSIDES FROM tRNA HYDROLYSATES BY TRACE ENRICHMENT ON AMBERLITE XAD-2

Neutralized hydrolysates (100 ml, each equivalent to 600 A_{260} units tRNA) were prepared as described in Experimental and applied to Amberlite XAD-2 columns (13 g, 30 × 0.9 cm I.D.).

Sample	*Cytokinin activity (μg KE/100 A_{260} units tRNA)**		
	E. coli	*Brewer's yeast*	*Wheat germ*
tRNA Hydrolysate	5.08	5.15	0.57
Sample flow-through from XAD-2 column	–**	–	–
Water-wash (10 bed volumes)	–	–	–
70% Ethanol eluate (bed volumes 1–5)	5.10	5.39	0.63
70% Ethanol eluate (bed volumes 6–10)	0.27	0.02	0.01
95% Ethanol eluate (bed volumes 11–15)	–	–	–

* Cytokinin activity is expressed as microgram kinetin equivalents (μg KE), as defined in Experimental.

** Bars indicate that cytokinin activity could not be detected in the tobacco callus bioassay.

may be due to the presence of residual charges on the resin as evidenced by the fact that the nucleotides could be quantitiatively recovered from the resin by elution with 70% ethanol containing 0.2 *M* potassium chloride (data not shown).

Recovery of cytokinins from tRNA hydrolysates

To test the effectiveness of neutral polystyrene resins in the quantitative recovery of cytokinins from more complex sources, the recovery of cytokinin-active ribonucleosides from tRNA hydrolysates was attempted using Amberlite XAD-2. Samples of wheat germ tRNA, *E. coli* tRNA, and yeast tRNA were hydrolyzed to ribonucleosides. The hydrolysates were applied to Amberlite XAD-2 columns, and the tobacco callus bioassay was used to monitor the recovery of cytokinin activity in various fractions from the columns. The results are shown in Table III. Cytokinin activity associated with the tRNA hydrolysates was retained by the Amberlite columns during sample application and a water wash. The cytokinin activity was quantitatively recovered from the columns by elution with ten bed volumes of 70% ethanol.

Recovery of cytokinins from A. tumefaciens culture medium

To test further the utility of neutral polystyrene resins in the recovery of cytokinins from natural sources, the efficiency of Amberlite XAD-2 in the quantitative recovery of cytokinin activity from *A. tumefaciens* cultures was examined. The results of this test are summarized in Table IV. Approximately 95% of the cytokinin activity detected in the culture medium was retained by the Amberlite XAD-2 column. The cytokinin activity retained by the column was recovered quantitatively by elution with 70% ethanol.

The cytokinin composition of the 70% ethanol eluate from the Amberlite XAD-2 column was examined by chromatography on a Sephadex LH-20 column in

TABLE IV

RECOVERY OF CYTOKININ ACTIVITY FROM *A. tumefaciens* C58 CULTURE MEDIUM

A total of 4700 ml of cell-free medium recovered from *A. tumefaciens* cultures as described in Materials and Methods was applied to an Amberlite XAD-2 column (175 g, 50 × 2.5 cm I.D.).

Sample	*Cytokinin activity** (*μg KE per liter culture medium*)
Culture medium (unfractionated)**	5.67
Sample flow-through from XAD-2 column**	0.34
Water-wash (10 bed volumes)	–***
70% Ethanol eluate (bed volumes 1–5)	5.83
70% Ethanol eluate (bed volumes 6–10)	0.05

* Cytokinin activity is expressed as microgram kinetin equivalents (μg KE) as defined in Materials and methods.

** The cell-free culture medium and the sample flow-through from the XAD-2 column were toxic when tested directly in the tobacco bioassay. Therefore, aliquots of these samples were taken to dryness *in vacuo* (37°C), and cytokinins were extracted from the dry solids with a 2:1 mixture of water-saturated ethyl acetate and *tert.*-butanol (six extractions per sample, 20 ml of solvent per extraction). The organic solvents were removed by evaporation *in vacuo*, and the extracts were tested in the tobacco callus bioassay.

*** Cytokinin activity could not be detected in the tobacco callus bioassay.

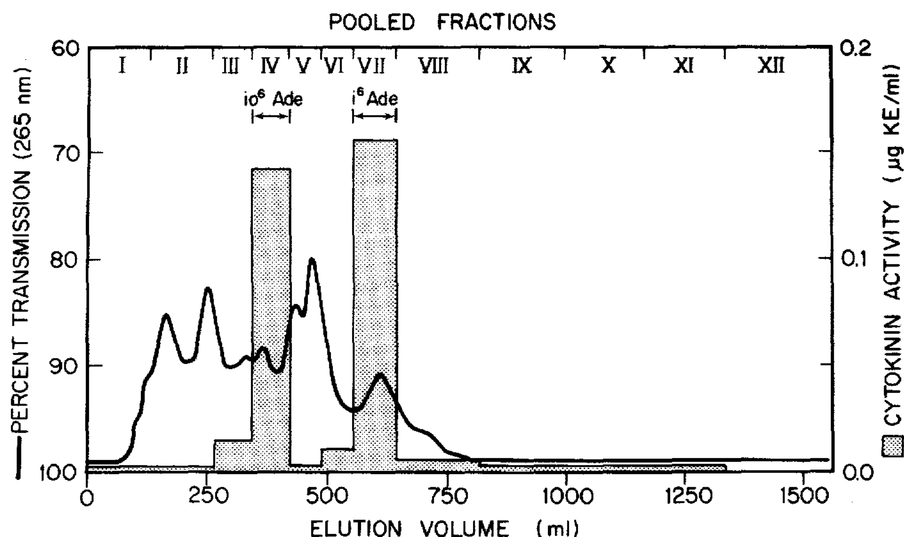

Fig. 1. Sephadex LH-20 fractionation of the cytokinin-active compounds recovered from *A. tumefaciens* C58 culture medium by trace enrichment on Amberlite XAD-2. The 70% ethanol eluate (bed volumes 1–10) recovered from the Amberlite XAD-2 column described in Table IV was taken to dryness *in vacuo* (37°C). The solids remaining after solvent removal were dissolved in 4.7 ml of 33% ethanol and applied to a Sephadex LH-20 column (60 ml, 45 × 2.5 cm I.D.) equilibrated with the same solvent. The column was eluted with 33% ethanol, and fractions (11 ml) were collected at a flow-rate of 35 ml/h. The fractions were pooled as indicated, and the ethanol was removed by evaporation *in vacuo*. Aliquots equivalent to 67% of each pooled fraction were tested for cytokinin activity in the tobacco callus bioassay as described in Materials and Methods. The cytokinin standards, zeatin (io^{6}Ade) and N^6-(Δ^2-isopentenyl)adenine (i^{6}Ade), were chromatographed on the same column immediately following elution of the experimental sample.

33% ethanol (Fig. 1). The elution profile from the Sephadex LH-20 column exhibited two major peaks of cytokinin activity, one corresponding to the elution position of io^{6}Ade (Fraction IV, 2.35 μg KE/liter medium) and the other to the elution position of i^{6}Ade (Fraction VII, 2.95 μg KE/liter medium). The cytokinin activity of these two fractions accounted for more than 88% of the total activity recovered from the *A. tumefaciens* C58 culture medium, which is in good agreement with the results of other studies with this strain[17].

DISCUSSION

The results reported here, together with the data of Stafford *et al.*[3], demonstrate that neutral polystyrene resins are effective in the recovery and trace enrichment of cytokinins from aqueous solutions. In the present study, all of the cytokinin bases and ribonucleosides tested were retained by Amberlite XAD-2 and by Porapak Q and were quantitatively recovered from the resins by elution with ten bed volumes of 70% ethanol. The recovery of cytokinin-active bases and ribonucleosides from complex natural sources (tRNA hydrolysates and media from *A. tumefaciens* cultures) also appeared to be quantitative. However, the behavior of i^{6}AMP indicates that care should be exercised in the application of the procedures described here to

systems where nucleotides or other charged forms of cytokinins may be of interest. The retention of adenine nucleotides by XAD-2 has been noted previously[22]. Further work would be needed to determine whether this property of the resin might have utility in cytokinin isolation procedures.

The ready availability of Porapak Q in fine mesh sizes makes this resin particularly well suited to applications where solution volumes must be kept as small as possible. The resin has a high capacity for cytokinins. As much as 34 mg of i^6Ado has been retained on 1 ml Porapak Q columns with no measurable appearance of UV absorption in the eluate. In studies of enzymes involved in cytokinin metabolism (unpublished), we have found small columns of Porapak Q to be particularly useful for the rapid removal of endogenous cytokinins from protein preparations. At appropriate ionic strength, there is no measurable retention of protein or loss of enzyme activity on the columns.

The course mesh sizes of commercial preparations of Amberlite XAD-2 somewhat limits the usefulness of the resin for applications involving the isolation of cytokinins. However, the resin Amberlite XAD-2 is inexpensive and exhibits rapid flow-rates under conditions of gravity-induced flow. It should be a convenient material for use in applications involving the processing of large volumes of solution. The possibility that some residual charges may be present on the resin should be considered in designing applications, and it is essential that the resin be adequately cleaned prior to use. The solvent extraction procedures used here were adequate for the purposes of this study, but for more critical applications, such as the isolation and chemical characterization of naturally occurring cytokinins, the resin should be prepared for use according to the procedures described by Loomis[4,5].

Both of the neutral polystyrene resins examined here appear to have utility as alternatives or supplements to the use of C_{18}-silica in the trace enrichment of cytokinins. The physical and chemical properties of these resins may offer advantages in particular applications.

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