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Chromatography of Cytokinins on a Neutral Polystyrene Resin

A SIMPLE PROCEDURE FOR THE SEPARATION OF THE *CIS* AND *TRANS* ISOMERS OF ZEATIN OR RIBOSYLZEATIN[1]

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ABSTRACT

A simple procedure for the separation of the *cis* and *trans* isomers of zeatin and ribosylzeatin by column chromatography on a neutral polystyrene resin, Porapak Q, in aqueous ethanol solutions is reported. The method has been used to examine the stereoisomer composition of ribosylzeatin isolated from wheat germ transfer RNA. Chromatographic data for several other cytokinins are also presented.

The identification of both the *cis* and *trans* isomers of zeatin [N^6-(4-hydroxy-3-methyl-2-butenyl)adenine] and ribosylzeatin [N^6-(4-hydroxy-3-methyl-2-butenyl)adenosine] as naturally occurring cytokinins (11, 13, 18) and constituents of tRNA molecules (5, 7, 20) has created a need for simple chromatographic methods of distinguishing between the stereoisomers of these compounds. Sephadex LH-20 chromatography (1) separates zeatin from ribosylzeatin, but it does not resolve the isomers of these compounds. The trimethylsilyl derivatives of the isomers can be separated by GLC (3). Playtis and Leonard (16) separated the *cis* and *trans* isomers of both zeatin and ribosylzeatin by TLC and used a modification of this procedure for the separation of the *cis* and *trans* isomers of isozeatin [N^6-(4-hydroxy-2-methyl-2-butenyl)adenine] by column chromatography (9). Partial resolution of *cis*- and *trans*-ribosylzeatin by partition chromatography on Celite column has been reported (3).

A simple procedure for the separation of the *cis* and *trans* isomers of zeatin and ribosylzeatin by column chromatography on a neutral polystyrene resin, Porapak Q, is reported here. This resin has found previous applications in both gas and liquid chromatography (8). In the latter case, it has been used for the separation and desalting of amino acids and peptides (14) and for the fractionation of steroids (4) and indole derivatives (15).

MATERIALS AND METHODS

Porapak Q was purchased from Waters Associates, Inc., Milford, Mass. Sephadex LH-20 is a product of Pharmacia. Wheat germ was kindly furnished by the VioBin Corporation, Monticello, Ill. Crude snake venom (*Crotalus adamanteus*) and alkaline phosphatase (calf intestinal mucosa, type VII) were obtained from Sigma Chemical Co. The sample of (±)-dihydrozeatin was kindly provided by Dr. J. Corse, United States Department of Agriculture Western Regional Research Laboratory, Berkeley, Calif. All other bases and ribonucleosides used in this study were kindly provided by Dr. Nelson J. Leonard (Department of Chemistry, University of Illinois, Urbana, Ill.). The synthesis of these compounds has been described (10, 16). Ethanol was distilled before use, and H_2O was double distilled from glass. Chromatographic solvents were degassed (reduced pressure) briefly before use.

Porapak Q Chromatography. Porapak Q (150–200 mesh) was prepared for use by suspending the resin in 95% ethanol for at least 1 hr. The resin was resuspended twice in aqueous ethanol at the concentration employed in chromatography and packed into 0.9 cm i.d. columns. Five grams of resin were used for each column. The bed volume varied slightly with the ethanol concentration, but averaged about 15 ml. A glass fiber disc was placed on top of the resin bed, and the packed columns were washed extensively with the chromatographic solvent (at least 50 ml per g resin) before use. Chromatographic samples (about 0.1 mg of each ribonucleoside and about 0.07 mg of each base) were dissolved in 1 ml of solvent for application to the column. The UV-absorption of the column eluate was monitored at 265 nm. Five-milliliter fractions were collected at a flow rate of about 10 ml/hr.

To optimize chromatographic separations it was important to minimize the dead space at the column outlet. Columns were equipped with a short capillary outlet ending in a Luer tip. A piece of nylon mesh placed over the tip and held in place by a Luer fitting served to support the resin bed. Teflon tubing was used to connect the Luer fitting to the flow cell of the UV-monitor.

Isolation of Ribosylzeatin from Wheat Germ tRNA. Wheat germ tRNA was prepared as described by Burrows *et al.* (5). The tRNA preparation was hydrolyzed to nucleosides with snake venom and alkaline phosphatase (6). The lyophilized hydrolysate was extracted with H_2O-saturated ethyl acetate and the ethyl acetate-soluble nucleosides were fractionated on a Sephadex LH-20 column in 35% ethanol as described by Armstrong *et al.* (1). The ribosylzeatin peak from the column was further purified by rechromatography on a Sephadex LH-20 column in distilled H_2O.

Cytokinin Bioassay. Column fractions were acid-hydrolyzed and tested for cytokinin activity in the tobacco bioassay (12, 17) as previously described (2). Cytokinin activity is expressed as μg kinetin equivalents (μg KE), defined as the micrograms of kinetin (6-furfurylaminopurine) required to give the same growth response as the test sample under the specified bioassay conditions.

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RESULTS

The *cis* and *trans* isomers of zeatin and ribosylzeatin were separated by chromatography on Porapak Q columns in 19% ethanol as shown in Figure 1. N^6-(3-hydroxy-3-methylbutyl)adenine, a compound formed by acid treatment of the cytokinin N^6-(Δ^2-isopentenyl)adenine [N^6-(3-methyl-2-butenyl)-adenine], eluted much earlier than zeatin under these conditions. Dihydrozeatin [N^6-(4-hydroxy-3-methylbutyl)adenine] eluted between the two zeatin isomers. Although *cis*- and *trans*-zeatin were completely resolved at this ethanol concentration, a better separation of the isomers of ribosylzeatin was obtained by chromatography on Porapak Q in 16% ethanol (See Fig. 4B). Chromatography of a commercial preparation of zeatin on Porapak Q in 16% ethanol (Fig. 2) revealed a significant contamination (18% of the total A_{270}) with a compound presumed to be the *cis* isomer.

Zeatin and ribosylzeatin are not separated by chromatography on Porapak Q in aqueous ethanol solutions, but the base and nucleoside forms of the more hydrophobic cytokinins are separated under these conditions (Fig. 3). Separation of the *cis* and *trans* isomers of 2-methylthiozeatin [2-methylthio-N^6-(4-hydroxy-3-methyl-2-butenyl)adenine] was not

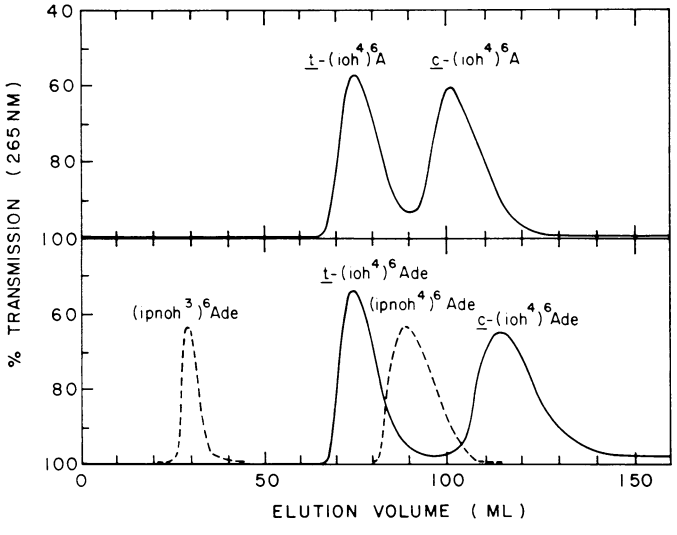

FIG. 1. Chromatography of zeatin and structurally related compounds on Porapak Q in 19% ethanol. Broken lines indicate compounds chromatographed separately on the same column. Abbreviations used: *c/t*-(ioh^4)^{6}A: *cis/trans*-ribosylzeatin; *c/t*-(ioh^4)^{6}Ade: *cis/trans*-zeatin; (ipnoh^3)^{6}Ade: N^6-(3-hydroxy-3-methylbutyl)adenine; (ipnoh^3)^{6}Ade: dihydrozeatin.

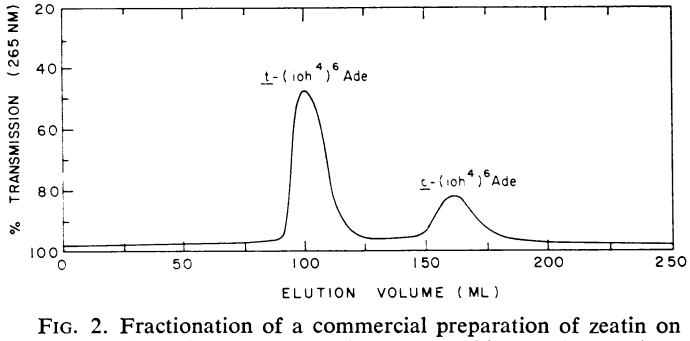

FIG. 2. Fractionation of a commercial preparation of zeatin on Porapak Q in 16% ethanol. The chromatographic sample contained about 0.34 mg of zeatin. Abbreviations used: *c/t*-(ioh^4)^{6}Ade: *cis/trans*-zeatin.

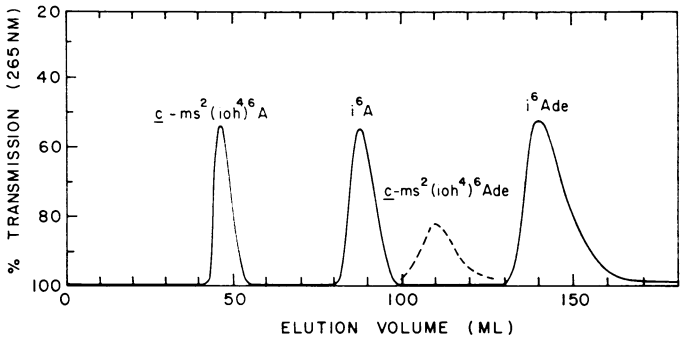

FIG. 3. Chromatography of cytokinins on Porapak Q in 38% ethanol. Broken lines indicate compounds were chromatographed separately on the same column. Abbreviations used: i^{6}Ade: N^6-(Δ^2-isopentenyl)adenine; i^{6}A: N^6-(Δ^2-isopentenyl)adenosine; *c*-ms^2(ioh^4)^6-Ade: *cis*-2-methylthiozeatin; *c*-ms^2(ioh^4)^{6}A: *cis*-2-methylthioribosyl-zeatin.

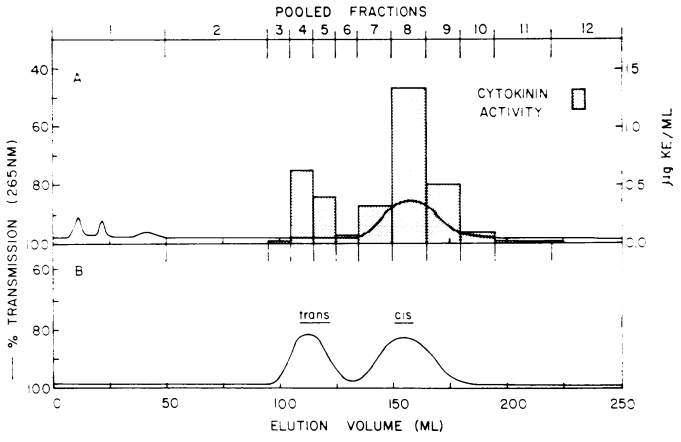

FIG. 4. Porapak Q chromatography of ribosylzeatin isolated from wheat germ tRNA. The chromatographic solvent was 16% ethanol. A: Chromatography of ribosylzeatin isolated from 5800 A_{260} units of wheat germ tRNA. Aliquots (50%) of the pooled fractions were tested for cytokinin activity in the tobacco bioassay. B: Chromatography of synthetic ribosylzeatin standards on the same column used for fractionation of the wheat germ tRNA sample. The standards were applied to the column after the fractionation of the wheat germ sample was completed.

achieved in this study, but the isomers of this compound can be separated by high pressure liquid chromatography on Porapak Q (D. L. Cole and N. J. Leonard, University of Illinois, personal communication).

A sample of ribosylzeatin isolated from an enzymatic hydrolysate of wheat germ tRNA was chromatographed on Porapak Q in 16% ethanol (Fig. 4). As expected from previous studies of the cytokinin constituents of wheat germ tRNA (5, 16), this compound chromatographed as a single UV-absorbing peak eluting at the position of *cis*-ribosylzeatin. However, bioassays of the column fractions revealed two peaks of cytokinin activity. In addition to the activity associated with *cis*-ribosylzeatin, an early peak of cytokinin activity was detected at the elution position of the *trans* isomer of ribosylzeatin. Although the available data are not sufficient to establish unequivocally the identity of this cytokinin-active compound, the failure to detect a peak of UV-absorption corresponding to the first peak of biological activity indicates the presence of a highly active cytokinin, and the *trans* isomer of zeatin, which is about 40 times more active than the *cis* isomer, is the plausible candidate. The presence of at least

small amounts of *trans*-ribosylzeatin in plant tRNA preparations has been reported by Vreman *et al.* (19, 20). The physiological significance of this finding is still uncertain.

The effects of factors other than ethanol concentration on the chromatographic behavior of cytokinins on Porapak Q columns have not been extensively investigated, but the inclusion of 0.01 N HCl in the aqueous ethanol solutions used for chromatography caused zeatin to elute at an earlier position in the profile. Trial experiments with other types of Porapak columns have indicated that better separation of the zeatin isomers is obtained on Porapak Q than on either the more hydrophobic Porapak P or more hydrophilic Porapak T.

DISCUSSION

The chromatography of zeatin and ribosylzeatin on Porapak Q columns provides a simple procedure for the separation of the *cis* and *trans* isomers of these compounds. The method is suitable for both preparative and analytical application and can be used for the purification of the individual stereoisomers from commercial preparations of zeatin or ribosylzeatin. An ethanol concentration of 16% is recommended for this purpose. The use of aqueous ethanol solutions permits easy purification and removal of the chromatographic solvents. The procedure can be adapted to high performance liquid chromatography systems (D. L. Cole and N. J. Leonard, University of Illinois, personal communication) where the use of higher pressures and smaller mesh sizes gives increased speed and resolution.

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