Cytokinins Extracted from Pinto Bean Fruit

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ABSTRACT

Extracts from various parts of Phaseolus vulgaris L. var. Pinto plants were found to exhibit cytokinin activity with the highest levels present in extracts of the fruit tissue. To separate the cytokinins present in the fruit, extracts were chromatographed in several solvent systems. Bioassays of chromatograms indicated the presence of active factors in those regions of migration associated with several known N-6-substituted aminopurines.

Ultraviolet and mass spectral studies confirm the presence of a mixture of N-6-substituted aminopurines in the active material isolated by cation exchange resin chromatography. The evidence strongly supports the conclusions that dihydrozeatin is naturally occurring as the free base and possibly as a ribonucleoside. Further, zeatin appears to occur mainly as a glycoside compound which is altered by KMnO₄ treatment and is hydrolyzed under acid conditions.

Previous investigations (3, 11) have indicated the existence of cytokinin activity in Pinto bean plants (Phaseolus vulgaris L. var. Pinto). The active factors detected appeared to be purine derivatives but were not characterized further. The purpose of this paper is to report in detail studies concerning the chemical characterization of the cytokinins present in extracts from Pinto bean fruit.

MATERIALS AND METHODS

Growth of Plant Materials. Plant parts used in preliminary experiments were derived from bean plants (Phaseolus vulgaris L. var. Pinto) grown under greenhouse conditions. The roots and primary leaves were harvested when the primary leaves were approximately 3.5 cm long.

For mass isolation studies, plants were grown under field conditions and the fruit was harvested when pods were about 3 to 4 inches in length.

Preparation of Extracts. Young primary bean leaves and roots as well as fruit were washed in tap water and macerated in a Waring Blender with sufficient 95% ethanol to produce a final 70% ethanolic extract. The debris was removed by filtering through cheesecloth and the filtrates are henceforth referred to as ethanolic extracts.

In most experiments, 70% ethanolic extracts were evaporated in vacuo at 50 °C and the residues, after reconstitution in twice distilled water, were washed three times with equal volumes of 1-butanol. The organic phases used in experiments are referred to as 1-butanol extracts.

Chromatographic Procedures and Solvents. Ascending paper chromatographic techniques involving the use of Whatman No. 1 filter paper were employed in most experiments and the migration of several pure standards was usually compared with that of the unknown materials. The chromatographic solvents used are as follows: water-saturated sec-butanol (WSB); 1-ethyl acetate-1-propanol-H₂O (4:1:2, v/v/v, upper layer); 1-butanol-concentrated NH₄OH-H₂O (3:1:1, v/v/v) (BAW); and 30 mm borate, pH 8.4 (B).

Tissue Culture Techniques and Assay. The soybean (Glycine max L. Merrill var. Acme) cotyledon callus bioassay developed by Miller (5, 7) served as the main assay system for all experiments. Basal medium for bioassays consisted of the following (mg/liter): KH₂PO₄, 300; KNO₃, 1,000; NH₄NO₃, 1,000; Cu(NO₃)₂·3H₂O, 0.35; Ca(NO₃)₂·4H₂O, 500; MgSO₄·7H₂O, 71.5; KCl, 65; MnSO₄·H₂O, 14; (NH₄)₆MoO₄·4H₂O, 0.1; KI, 0.75; ZnSO₄·7H₂O, 3.80; H₂BO₃, 1.6; myo-inositol, 100; nicotinic acid, 0.5; pyridoxine-HCl, 0.2; thiamine-HCl, 0.2; Na,EDTA, 13.4; FeSO₄·7H₂O, 9.9; sucrose, 30,000; Bactoagar, 10,000; α-naphthalene acetic acid, 2.0; and, in some cases, kinetin at 0.5 mg (for controls).

After adjusting the pH to 5.8 with NaOH, the medium, which was usually prepared in multiple strength, was divided into equal portions and supplemented with plant extracts or portions of chromatograms corresponding to specific Rf regions. The pH was then readjusted after the medium was brought to single strength with twice distilled water. The agar was melted and 50-mL aliquots were poured into 125-mL Erlenmeyer flasks, which were autoclaved at 15 psi and 121 °C for 15 min.

Three pieces of soybean callus tissue were planted in each flask of hardened medium. Cultures were maintained at 30 °C under constant illumination (about 40 ft-c) for 28 days and then weighed.

Mass Isolation and Purification of the Active Component(s) Present in Pinto Bean Fruit. Approximately 58 kg of Pinto bean fruit were extracted in enough 95% ethanol to produce a final 70% ethanolic extract. This material was then subjected to a Dowex AG 50W-X8 H⁺ (50–100 mesh) cation exchange column chromatographic procedure presented in Table I, which is similar to the early methods of Miller (5) for the isolation of zeatin.

Ultraviolet and Mass Spectral Studies. Ultraviolet spectral studies involving isolated material were performed on a Beckman DB-5 recording spectrophotometer under acidic (0.1 N HCl), basic (0.1 N NaOH), and neutral (twice distilled H₂O) conditions.

1 Contribution No. 60 from the Department of Biology, The Pennsylvania State University, and No. 3867 from the Pennsylvania Agricultural Experiment Station.

2 Abbreviations: B: borate; BAW: 1-butanol-ammonia-water; D-Z: dihydrozeatin; 2iP: 6-γ,α-dimethylallyl aminopurine; WSB: water-saturated sec-butanol; Z-R: zeatin ribonucleoside.
Mass spectral data was obtained from an AEI/MS-9 mass spectrometer at high resolution (70 ev).

RESULTS AND DISCUSSION

Preliminary experiments were initiated to determine the relative distribution of cytokinin activity within various plant parts. 1-Butanol extracts equivalent to 25 g fresh weight of primary leaf, root, and fruit tissues were prepared from 70% ethanolic Pinto bean extracts, streaked on Whatman No. 1 filter papers, and developed in the BAW solvent. Chromatograms were divided into four major Rf regions (0.0—0.5, 0.5—0.7, 0.7—0.9, 0.9—1.0), incorporated into soybean medium, and bioassayed for activity. The results (Fig. 1) indicate the relative levels of cytokinin activity within the three plant parts tested. Although the response produced by leaf and whole root extracts is low compared with that of the fruit, it is evident that the peak migration of the active material (Rf 0.7—0.9) is similar for the leaf, root, and fruit extracts.

Because the levels of cytokinin activity appeared to be significantly higher in Pinto bean fruit, this plant part represented the best source of material for use in characterization studies. Ethanolic extracts of the fruit, in various concentrations, were evaporated to dryness and the residues were bioassayed for activity (Fig. 2). As illustrated, the factor(s) present in the fruit extract is active over a wide concentration range.

For characterization of the factors responsible for stimulating callus proliferation, conventional paper chromatographic techniques were employed. Separate 1-butanol extracts equivalent to 25 g (unless otherwise specified) of Pinto bean fruit were streaked on Whatman No. 1 filter paper and developed in several solvent systems. The migration of the active components in the extract was compared with that of zeatin, dihydrozeatin (D-Z), 6-γ,γ-dimethylallyl aminopurine (2iP), and zeatin ribonucleoside (Z-R) standards.

A growth analysis of chromatograms developed in ethyl acetate-1-propanol-water revealed three peaks of activity (Fig. 3). As illustrated, a portion of the 1-butanol extract migrated to regions on chromatograms associated with the migration for Z-R, zeatin, and D-Z. On the other hand, the activity observed at Rf region 0.0 to 0.2 does not correspond to the migration of any of the standards used.

A growth study of chromatograms developed in WSB solvent (Fig. 4) indicates the presence of cytokinin at Rf regions close to those for the migration of N-6-substituted purine free base and nucleoside cytokinins (Rf 0.70—0.95). This finding is consistent with the recent work of Miura and Miller (10). In addition, when the activity found at Rf regions 0.70 to 0.95

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**Table I. Flow Diagram for the Isolation of Cytokinins from Pinto Bean Fruit Extracts**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>70% ethanolic extract of fruit</td>
</tr>
<tr>
<td>2</td>
<td>Debris removed by filtering through cheesecloth</td>
</tr>
<tr>
<td>3</td>
<td>Dowex AG 50W-X8 H⁺ (50—100 mesh) column (500 ml bed volume)</td>
</tr>
<tr>
<td>4</td>
<td>H₂O wash (500 ml)</td>
</tr>
<tr>
<td>5</td>
<td>Elution with 6 N NH₄OH (1500 ml)</td>
</tr>
<tr>
<td>6</td>
<td>Ammonia removed by vacuum distillation</td>
</tr>
<tr>
<td>7</td>
<td>Acidification to pH 1.5 with HCl</td>
</tr>
<tr>
<td>8</td>
<td>Precipitates removed by centrifugation (5000g; 5 min)</td>
</tr>
<tr>
<td>9</td>
<td>Dowex AG 50W-X8 H⁺ (50—100 mesh) column (35 ml bed volume)</td>
</tr>
<tr>
<td>10</td>
<td>H₂O wash (600 ml)</td>
</tr>
<tr>
<td>11</td>
<td>Elution with 4 N HCl (350 ml)</td>
</tr>
<tr>
<td>12</td>
<td>Eluate diluted to 1 N HCl</td>
</tr>
<tr>
<td>13</td>
<td>Dowex AG 50W-X8 H⁺ (50—100 mesh) column (35 ml bed volume)</td>
</tr>
<tr>
<td>14</td>
<td>H₂O wash (300 ml)</td>
</tr>
<tr>
<td>15</td>
<td>Elution with 6 N NH₄OH (300 ml)</td>
</tr>
<tr>
<td>16</td>
<td>Eluate evaporated in vacuo to remove ammonia</td>
</tr>
<tr>
<td>17</td>
<td>Solids reconstituted in 95% ethanol</td>
</tr>
<tr>
<td>18</td>
<td>Paper chromatography</td>
</tr>
</tbody>
</table>

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**Fig. 1. Bioassay of chromatograms of 1-butanol Pinto bean leaf, root, and fruit extracts. The solvent used was t-butanol-concentrated NH₄OH-H₂O (3:1:1, v/v).**

**Fig. 2. Bioassay of a 70% ethanolic extract of Pinto bean fruit at various concentrations.**
The other readjusted of volume streaked on residues in ethanol, and divided to the RF chromatographed upper equivalent whereas water-saturated was peak corresponds (4:1:2, and (8, 10), to g eq 0.93-0.98, 0.96-0.98, and 0.80-0.88, respectively.

was eluted and rechromatographed in 30 mM borate, pH 8.4 (8, 10), two peaks of activity were observed (Fig. 5). The lower peak corresponds to a region associated with purine free base migration whereas the upper peak may be correlated with nucleoside migration.

For substantiation of the fact that the factor present in the upper region of chromatograms developed in solvent B might be a purine nucleoside, acid hydrolysis was performed. After chromatographing a 50-g eq of 1-butanol fruit extract in WSB, the RF region 0.70 to 0.95 was eluted in 95% ethanol and streaked on papers which were developed in solvent B. The 0.6 to 1.0 RF regions of the borate chromatograms were eluted in ethanol, dried down, reconstituted in twice distilled water, and divided into two 25-g aliquots. To one portion, an equal volume of 0.2 N HCl was added to make a 0.1 N HCl solution. The other portion remained as a control. Both solutions were placed in a 100 C water bath for 1 hr, cooled in an ice bath, readjusted to pH 7.0 with NaOH, and evaporated to dryness. The residues were reconstituted in 95% ethanol, chromatographed in solvent B, and bioassayed. It is evident (Fig. 6) that control activity is present mainly in the upper nucleoside regions with some activity in lower free base ranges. The latter is due to traces of material present in the 0.6 RF region when chromatograms were cut for elution as mentioned previously. However, after acid treatment, which presumably cleaves the glycosidic link, the activity shifts to the lower RF regions, indicative of free base migration.

In other experiments, the 0.70 to 0.95 RF region of WSB chromatograms was eluted in ethanol and rechromatographed in solvent B. After the regions corresponding to free base (RF 0.0-0.6) and nucleoside (RF 0.6-1.0) were eluted, both eluates were subjected to aqueous KMnO₄ treatment (4, 6, 9), dried, and incorporated directly into basal medium. Results of the bioassay (Fig. 7) shows that the free-base activity is not appreciably affected by KMnO₄ treatment, whereas the nucleoside-like material, although not completely labile to the oxidation, still exhibited a 40% decrease.

**Fig. 3.** Bioassay of chromatograms developed with ethyl acetate-1-propanol-H₂O (4:1:2, v/v, upper layer). Approximately 25 g eq of 1-butanol Pinto bean fruit extract were chromatographed. The migration of zeatin, D-Z, 2iP, and Z-R standards corresponded to RF regions: 0.64–0.77, 0.73–0.81, 0.94–0.98, and 0.45–0.59, respectively.

**Fig. 4.** Bioassay of chromatograms of 1-butanol Pinto bean fruit extract equivalent to 25 g fresh weight of tissue. The solvent used was water-saturated sec-butanol. The migration of zeatin, D-Z, 2iP, and Z-R standards corresponded to RF regions: 0.87–0.93, 0.93–0.98, 0.96–0.98, and 0.80–0.88, respectively.

**Fig. 5.** Bioassay of chromatograms developed in 30 mM borate, pH 8.4. Approximately 25 g eq of 1-butanol Pinto bean fruit extract were first chromatographed in water-saturated sec-butanol, and the compounds of 0.70–0.95 RF region were eluted and rechromatographed in borate. The migration of zeatin, D-Z, 2iP, and Z-R standards corresponded to RF regions 0.49–0.58, 0.53–0.60, 0.39–0.49, and 0.86–0.91, respectively.

**Fig. 6.** Bioassay of chromatograms of presumed nucleoside activity subjected to acid hydrolysis. A portion of the active material eluted from 0.60–1.0 RF region of borate chromatograms was treated with 0.1 N HCl at 100 C for 1 hr and rechromatographed in 30 mM borate, pH 8.4. The migration of zeatin, D-Z, 2iP, and Z-R standards corresponded to RF regions 0.49–0.58, 0.53–0.60, 0.39–0.49, and 0.86–0.91, respectively.
Since it is well known that compounds such as zeatin, which contain an N-6-unsaturated substituent, are labile to K\textsubscript{MnO\textsubscript{4}} treatment and yield oxidation products which are biologically inactive at lower concentrations (4, 6, 9) the above results suggest that the predominant naturally occurring free-base-like cytokinin in fruit extracts is composed of a saturated substituent. Although not illustrated, pure samples of zeatin and 2iP were completely oxidized by the same procedure whereas pure dihydrozeatin remained unchanged. The results also suggest that at least two cytokinins, one of which appears to be K\textsubscript{MnO\textsubscript{4}} labile, migrate to the nucleoside region of chromatograms.

Because the active factors detected in fruit extracts were chromatographically similar to the N-6-substituted aminopurines, a conventional isolation technique was employed to obtain sufficient material for further analysis. Approximately 58 kg of Pinto bean fruit were extracted in enough ethanol to produce a 70% ethanolic extract. After removal of debris by filtration through cheesecloth the material was subjected to the Dowex AG50 W-X8 H\textsuperscript{+} cation exchange resin chromatography procedure presented in Table I. The final ammonia eluate was then chromatographed in the BAW solvent system and 0.8 to 1.0 mg of material active in the soybean cotyledon callus bio-

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Maximal (max) and Minimal (min) Absorption at Wavelengths Indicated</th>
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<tbody>
<tr>
<td></td>
<td>Neutral (H\textsubscript{2}O)</td>
</tr>
<tr>
<td></td>
<td>max</td>
</tr>
<tr>
<td>Zeatin</td>
<td>269</td>
</tr>
<tr>
<td>Dihydrozeatin</td>
<td>267</td>
</tr>
<tr>
<td>6-γ,7-Dimethylallyl aminopurine</td>
<td>269</td>
</tr>
<tr>
<td>Unknown</td>
<td>268</td>
</tr>
</tbody>
</table>

Fig. 7. Bioassay of materials eluted from the 0.0–0.60, 0.60–1.0 R\textsubscript{f} regions of chromatograms developed in the borate solvent and subjected to K\textsubscript{MnO\textsubscript{4}} treatment. The migration of zeatin, D-Z, 2iP, and Z-R standards corresponded to R\textsubscript{f} regions 0.49–0.58, 0.53–0.60, 0.39–0.49, and 0.86–0.91, respectively.

Fig. 8. Ultraviolet spectra of the active material from Pinto bean fruit under acidic, basic, and neutral conditions.

Fig. 9. Mass spectrum of the active material from Pinto bean fruit.

assay was detected on chromatograms as a well defined ultraviolet-quenching band.

The active factor(s) was cochromatographed in the WSB solvent systems with pure standards and was found to migrate closely to trans-6-(4-hydroxy-3-methylbut-2-enyl)-aminopurine (zeatin) and 6-(4-hydroxy-3-methylbutyl)-aminopurine (dihydrozeatin). Also, treatment of the unknown with aqueous
lated product conditions. This pattern is somewhat consistent with that of free base zeatin according to our experiments with pure zeatin and to experiments of Miller (6, 9). Unlike the zeatin standard, however, after KMnO₄ treatment of the unknown, an ultraviolet-quenching spot was still detected at the point of migration associated with unoxidized compound in addition to the lower spots. These results suggest that at least two chromatographically similar, but chemically distinct, components were present in the isolated product.

The ultraviolet absorption spectra of the isolated active material under neutral, acid (0.1 N HCl), and basic (0.1 N NaOH) conditions (Fig. 8) are suggestive of N-6-substituted adenines. However, a comparison of the spectral properties of the isolated product with those of three cytokinin standards (Table II) indicated that the isolated material consisted of more than one adenine derivative. Furthermore, in consideration of the acid conditions prevailing during the cation exchange separation procedure and from the data obtained in the chromatographic studies, it is very likely that the isolated fraction actually represented a mixture of the naturally occurring free base, amounts of free base recovered from the presumed hydrolysis of natural cytokinin nucleoside(s), and possibly small amounts of intact cytokinin ribonucleosides. This idea is given added support by the following mass spectral data.

The mass spectrum of the isolated active product exhibits prominent peaks characteristic of the free base zeatin at m/e 219, 202, 188, 160, 149, 148, 136, 135, 119, and 108 (Fig. 9). Also, ions corresponding to those of dihydrozeatin are evident at m/e 221, 204, 190 and 162. Further, the peaks from m/e 149 to 108 already mentioned for zeatin are characteristic of dihydrozeatin as well. Thus, the over-all fragmentation pattern of the respective side chains and adenine provides strong evidence for the presence of zeatin and dihydrozeatin.

Although not conclusive, the mass spectrum of the isolated product does suggest the presence of nucleoside-like material. For example, the peaks at m/e 353 (M), 264 (M-89), and 250 (M-103) are indicative of dihydrozeatin ribonucleoside. The ions, M-89 and M-103, are characteristic of ribonucleosides and arise from the fragmentation of the ribose portion of the nucleoside (1). In addition, there is a prominent peak at m/e 133, which very likely represents ribose.

Even though the molecular ion for zeatin ribonucleoside (M = 351) is not evident, the peaks at m/e 262 (M-89) and 248 (M-103) and the fragmentation pattern (2) to adenine (m/e 135) are indicative of this compound. However, this idea is not conclusive and further speculation concerning the chemical nature of the presumed nucleosides and other aspects of the spectrum are not justified in the absence of pure compounds.

CONCLUSIONS

The chromatographic, ultraviolet, and mass spectral data provide sufficient evidence to conclude that the cytokinins present in extracts of Pinto bean fruit are N-6-substituted aminopurines. It is also reasonable to conclude that the active material isolated by cation exchange resin chromatography contained a mixture of substances including the free base zeatin, dihydrozeatin, and possibly ribonucleosides. In addition, it is very likely that zeatin does not occur appreciably in the fruit as the free base but rather is present in the isolated material as a conversion product arising from the acid hydrolysis of a zeatin glycoside. On the other hand, the paper chromatographic and bioassay data indicate that the naturally occurring free base is dihydrozeatin or a highly similar adenine compound with a saturated substituent that is not labile to KMnO₄ treatment. Also, the presence of dihydrozeatin in the active material isolated by cation exchange resin chromatography is indeed substantiated by the mass spectrum.

There is a great deal of circumstantial evidence favoring the presence of cytokinin ribonucleosides in Pinto bean fruit extracts. Further experiments are now in progress to determine their exact chemical nature.

Acknowledgments—We appreciate the technical assistance provided by Drs. Richard D. Schein and Robert H. Hamilton. We are especially indebted to Dr. Roy G. Creech for providing the large quantities of bean fruit used in the mass isolation studies and to Dr. D. S. Latham for his generous donation of zeatin and zeatin ribonucleoside.

LITERATURE CITED