Isolation of Indole-3-Acetyl Amino Acids using Polyvinylpolypyrrolidone Chromatography

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ABSTRACT

Amino acid conjugates of IAA can be chromatographed on polyvinylpolypyrrolidone (PVPP) using either acidic methanol or aqueous buffers as eluents. In the aqueous system, elution of the compounds is pH-dependent, and the pattern obtained suggests that hydrophobic interactions contribute substantially to the chromatographic behavior of IAA peptides on PVPP. Purification of soybean seed extracts by PVPP chromatography produced fractions containing indole-3-acetylglutamate and indole-3-acylglutamate, based on chromatographic and mass spectral analysis, as well as three other indolic compounds, tentatively identified as N-acyl tryptophan derivatives. PVPP chromatography provides an effective preliminary purification of IAA peptides from plant extracts prior to their separation by other techniques such as high performance liquid chromatography.

Higher plants metabolize IAA to a variety of conjugates, including IAA-glucose, IAA-inositols, IAA-glucans, and IAA-amino acids (7). The most widely studied amino acid conjugate has been IAAsp,2 the compound having been identified both as a product formed from exogenous IAA (2, 23, 26, 28) and as a naturally occurring plant substance (1, 6, 20). Synthetic auxins are also converted to aspartate conjugates in plant tissues (11, 29).

The conversion of auxins to conjugates with amino acids other than aspartate has also been reported. 2,4-D is converted to a number of amino acid conjugates in soybean plants and tissue cultures (12), and Brenner and Tonkinson (5) showed that soybean plants will also metabolize 1-naphthaleneacetic acid to 1-naphthylacetylglutamate. Similar compounds are also formed from IAA. Thurman and Street (27) first reported the formation of IAGlu in tomato seedlings, Hollenberg et al. (15) have demonstrated the synthesis of both IAAsp and IAGlu from IAA in cucumber seedlings, and Bogers et al. (4) detected the conversion of IAA to a conjugate containing both aspartate and glutamate. Crown gall tissue derived from Parthenocissus tricuspidata produces five different amino acid conjugates from IAA (10). On the other hand, to my knowledge, there are only three reports for the natural occurrence of an IAA amino acid other than IAAsp (8, 22, 24).

One limiting factor in obtaining information concerning the natural occurrence of IAA peptides is the availability of simple, rapid purification procedures for the compounds. PVPP chromatography has been employed to isolate a number of plant hormones (13), primarily because of its capacity to bind phenolic compounds through hydrogen bonding (16). Nonphenolic aromatic compounds, including indoles, also adsorb to PVPP, apparently through hydrophobic interactions (21), and this allows for additional purification of these substances. Although PVPP chromatography has been employed previously in IAA peptide isolations (8, 24), its use for this purpose has not been systematically described. I show here that IAA peptides can be chromatographed on PVPP, both in aqueous buffers and in acidic methanol, and that these techniques can be used to isolate IAAsp and IAGlu from soybean seeds. This tissue is known to contain most of its IAA in peptide bond forms (3), with IAAsp accounting for approximately one-half of the total seed IAA (6). Chromatographic evidence for the presence of IAGlu in these seeds has also been presented (8, 22). A preliminary report of these results appeared previously (22).

MATERIALS AND METHODS

Plant Material. Seeds of Glycine max L. cv Hark were obtained from Dr. Jerry Cohen, Plant Hormone Laboratory, USDA/ARS, Beltsville Agricultural Research Center, Beltsville, MD 20705 (original source, Committee for Agricultural Development, Iowa State University).

Chemicals. [2-14C]IAAAsp and [2-14C]IAGlu (42.2 μCi/μmol) were a gift from Dr. Cohen, and unlabeled IAHis and IAVal were obtained from Dr. W. K. Purves, Biology Department, Harvey Mudd College, Claremont, CA 91711. Other IAA peptides were synthesized for use as chromatographic standards, either by the method of Mollan et al. (18), or by carbodiimide coupling of IAA to amino acid ethyl esters, followed by hydrolysis of the resulting peptide ethyl esters. As an example of the latter method, IAGlu was prepared by mixing 100 mg IAA and 400 mg L-glutamate diethyl ester HCl in 20 mL 50% acetone and adding 384 μg EDAC. The mixture was stirred at room temperature, maintaining pH between 4.5 and 5.5, until no further pH changes occurred. Acetone was removed in vacuo at 45°C, and the aqueous residue was made 1 M in NaOH and kept overnight in a 45°C incubator. The hydrolysate was adjusted to pH 1.5 with 6 N HCl and partitioned three times with 3 volumes ether, the ether phases were dried in vacuo, and the residue was dissolved in ethanol and purified by silica gel TLC using chloroform-methanol-acetic acid (75:20:5) for development.

IAA peptide methyl esters were prepared from the free acids in 0.1 M EDAC in methanol at 45°C. After 3 h, the methyl esters were partitioned into ether and further purified by silica gel TLC in chloroform-methanol (95:5).

PVPP and organic compounds other than solvents were from

1 Supported by a grant from the Research Corporation of America and by a faculty development grant from Westmont College.

2 Abbreviations: IAAla, IAAsp, IAGlu, IAHis, IAVal, the N-indole-3-acetyl derivatives of alanine, aspartate, glutamate, histidine, phenylalanine, tyrosine, and valine, respectively; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; MA, 1% acetic acid in methanol; m/z, mass to charge ratio; PVP, polyvinylpyrrolidone; PVPP, polyvinylpolypyrrolidone; Vb, column bed volume; Ve, elution volume.
Sigma Chemical Co. All other materials were reagent grade from various suppliers. Methanol, pyridine, and ethyl acetate were redistilled before use.

**PVPP Chromatography.** PVPP was washed and freed from fines by repeated suspension and sedimentation in either water or methanol, depending on the elution conditions. Two additional washes with elution solvent were given before packing the column, and the column was washed with an additional two bed volumes of solvent before applying the sample. Samples were applied in the elution solvent (volumes up to 20% of the column volume) and eluted using a peristaltic pump to produce flow rates of 25 to 30 ml/h for 1.0 cm i.d. columns and 60 ml/h for a 1.5 cm i.d. column. Fractions were collected and analyzed for A at 280 nm or for radioactivity.

**Extraction of IAAp and IAGlu from Soybean Seeds.** Soybean seeds were ground to 20 mesh in a Wiley mill, and 200 g meal was extracted three times with 600 ml 70% acetone at 4°C with filtration through Whatman No. 1 paper between extractions. The final residue was washed with 200 ml 70% acetone in the filter. [14C]IAAsp and [14C]IAGlu (0.12 μCi each) were added to the final extraction to act as tracers. The combined filtrates were reduced in vacuo at 45°C to a golden brown syrup, washed out of the evaporating flask with water (100 ml final volume), and adjusted to pH 3 with 12 N HCl. The acidified extract was partitioned five times with 300 ml ether, and the ether phases were pooled and partitioned three times with 35 ml 1% aqueous pyridine:pyridine with the phases of the final extraction being separated by centrifugation. The aqueous phases were pooled and taken to dryness in vacuo at 45°C. The residue was dissolved in 10 ml 1% methanolic acetic acid, redried to remove pyridine, and redissolved in 3 ml MA.

The sample was applied to a 1.5 × 19 cm column of methanolic PVPP, and eluted with MA, collecting 10 ml fractions. Radioactivity in 50 μl aliquots of fractions was measured by liquid scintillation counting in ACS (Amersham), and material eluting between 100 and 150 ml was pooled and evaporated to near dryness in vacuo at 45°C. The residue was dissolved in 5 ml methanol, 3 ml 50 mM pyridinium acetate (pH 4.3) was added, and the methanol was removed in vacuo. The aqueous sample was applied to a 1.0 × 23 cm column of PVPP, equilibrated with the same buffer, and eluted at 0.5 ml/min, collecting 2.5 ml fractions. Fractions were tested for radioactivity, and material eluting between 32 and 53 ml was pooled and dried. The residue was dissolved in methanol and redried twice to remove pyridinium acetate and finally dissolved in 100 μl methanol.

The sample was subjected to reversed phase HPLC (4.6 × 250 mm 10 μm Alltech C18 column in a Cole Scientific gradient-forming HPLC) in three 20 to 40 μl aliquots. The flow rate was 1 ml/min, and the sample was eluted with 1% acetic acid (5 min), followed by a linear methanol gradient to 40% methanol in 4 min and holding at this composition for an additional 20 min. The column effluent was monitored at 280 nm, and 0.5 ml fractions were collected and tested for radioactivity. Under these conditions, the retention times of IAAp and IAGlu were 20.2 and 21.4 min, respectively. Based on the radioactivity of the pooled IAAp and IAGlu fractions, the yield to this point was 29%.

The sample was prepared for analysis by GC and GC-MS by methylation in 0.5 ml methanolic 0.1 M EDAC, as described above. The ether phases from solvent partitioning were dried and redissolved in 60 μl ethyl acetate. Yield for the methylation procedure was 60%.

**GLC and GC-MS.** Methylated samples were chromatographed isothermally at 270°C through a 2 mm × 2 m glass column of 3% OV-17 on Anakrom AS (Analabs) using a Perkin-Elmer Sigma 3B gas chromatograph interfaced with a Hewlett-Packard 3390A recording integrator. Helium carrier gas flow was 20 ml/min, the sample was injected at 270°C, and detection was either by flame ionization or with a nitrogen phosphorous detector. Under these conditions, the retention times for bis-methyl-IAAsp and bis-methyl-IAGlu were 5.1 and 7.1 min, respectively, with a minor peak from bis-methyl-IAGlu appearing at 6.2 min (Fig. 3).

GC-MS analysis was performed with a Hewlett-Packard 5992A GC-MS System, using a 0.3 mm × 25 m fused silica capillary column with a 0.5 μm coating of OV-1. The injection port was at 240°C, helium carrier gas flow as 1 ml/min, and the oven temperature was programmed from 90 to 270°C at 6°C/min. Ionizing voltage was 70 eV, and the electron multiplier was run at 1400 V. Retention times for bis-methyl-IAAsp and bis-methyl-IAGlu were 18.4 and 20.2 min, respectively.

**RESULTS AND DISCUSSION**

**PVPP Chromatography of IAA Peptides.** Chromatography of IAA peptides on PVPP in aqueous buffers is pH-dependent. Table I presents the elution volumes for IAA and for several IAA peptides. As shown, adsorption of most of the compounds to PVPP increased with decreasing pH, suggesting that maximum adsorption to the column depends on a substance being unionized. This pattern is the same as that observed in PVPP chromatography of simple indoles (21). Compared to IAA, more acidic buffers are required for strong adsorption of the peptides to the column, an effect attributable to the more acidic carboxyl groups of the amino acid conjugates.

Some separation between peptides occurs, based on the properties of their amino acid side chains. The basic amino acid conjugates, exemplified by IAHis, always carry a net charge and, therefore, elute rapidly from the column, independent of buffer pH. The chromatography of the acidic and neutral amino acid conjugates depends on the relative polarities of their side chains at the pH of the elution buffer. The most strongly adsorbed peptides are the conjugates of the aromatic amino acids, and IATyr is further retarded in its elution by hydrogen bonding to the column through its phenolic hydroxy group.

Based on sulfuric acid charring of thin layer chromatograms of column fractions, most of the material in plant extracts elutes from PVPP within two bed volumes. Thus, with an appropriate choice of buffer pH, PVPP chromatography can result in a substantial purification of IAA peptides as a group from plant extracts.

IAA peptides can be partially purified from plant extracts by solvent partitioning (6), although the organic solvent-soluble fractions obtained can be difficult to chromatograph using aqueous buffers as described above. A solution to this general problem was suggested by Mousdale and Knee (19) who showed that methanol could be used as an eluent for PVPP chromatography. However, when this method was tested with IAAsp, the

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**Table I. PVPP Chromatography of IAA and IAA Peptides**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Vv/Vo</th>
<th>pH 5</th>
<th>pH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>5.4</td>
<td>17.2</td>
<td></td>
</tr>
<tr>
<td>IAAla</td>
<td>1.9</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>IAAsp</td>
<td>1.6</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>IAGlu</td>
<td>1.6</td>
<td>10.6</td>
<td></td>
</tr>
<tr>
<td>IAHis</td>
<td>1.6</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>IAPhe</td>
<td>4.3</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>IATyr</td>
<td>7.5</td>
<td>43.9</td>
<td></td>
</tr>
<tr>
<td>IAVal</td>
<td>2.3</td>
<td>7.2</td>
<td></td>
</tr>
</tbody>
</table>
compounds were eluted in a diffuse peak (Fig. 1). Addition of 1% acetic acid to the eluent produced a much sharper peak with a somewhat greater retention volume. Similar results were obtained with IAA when the pure compound was chromatographed in methanol, but only rarely in separations of plant extracts (data not shown). In the latter case, it seems likely that the other organic acids in the sample are able to substitute for acetic acid in the eluent to produce the observed chromatographic behavior. IAA and IAAsp were eluted in 2.7 and 3.8 bed volumes, respectively, when chromatographed in acidic methanol. These values are smaller than those obtained in acidic aqueous buffers, supporting the concept that hydrophobic interactions play a large part in determining the chromatographic behavior of indoles on PVPP. The mechanism by which acetic acid affects the chromatography of these compounds in methanol is unclear, however. Acetic acid (pK = 4.75) is not strong enough to protonate the IAAsp carboxyl groups (pK₁ = 1.88; pK₂ = 3.65) if dissociation of the compound were to be the sole cause of the observed peak broadening.

In the final isolations of IAAsp and IAGlu done for these studies, methanic and aqueous PVPP chromatography were performed in series, as described in “Materials and Methods.” This takes advantage of the somewhat different separations achieved in the two systems. For example, the order of elution for IAA and IAAsp in acidic methanol is reversed compared to that in pH 3 buffer. Samples, prepared by this procedure, were sufficiently pure for further separation by HPLC, and the general approach has also proved suitable for purifying IAA from seed extract hydrolysatapes (data not shown).

Isolation of Soybean Seed IAA Peptides. A group purification of the seed IAA peptides was attempted using aqueous PVPP chromatography. The aqueous residue from 70% acetone extract was chromatographed at pH 3, pooling fractions eluting between 2.5 and 12 bed volumes, and then at pH 5, collecting material eluting between 0.5 and 3 bed volumes. After further purification by partitioning into ether at pH 1.5, the sample was analyzed by silica gel TLC in chloroform-methanol-acetic acid (70:20:5). Five indolic substances were revealed in the chromatogram upon spraying with Van Urk’s reagent (25) with Rf values of 0.19, 0.27, 0.37, 0.45, and 0.53 (compounds 1–5, respectively). Preparations of the five compounds were obtained by TLC and hydrolyzed in 7 N barium hydroxide. Analysis of the hydrolysates by TLC in n-butanol-acetic acid-water (65:13:22) showed compounds 1, 4, and 5 to be tryptophan derivatives (Fig. 2). Given their solubility in ether at low pH, their chromatographic properties, and their conversion to tryptophan in alkaline hydrolysis, compounds 1, 4, and 5 appear to be N-acetyl tryptophan derivatives, resembling N-malonyl-D-tryptophan, first identified by Good and Andreac (14).

**Figure 1.** Methanolic PVPP chromatography of IAAsp in the absence (O—O) and the presence (●—●) of 1% acetic acid. The fractions were 1.9 ml, and Vₐ was 10.5 ml.

**Figure 2.** Silica gel TLC of intact and base-hydrolyzed (H) indolic compounds 1-5, isolated from soybean seeds. The chromatogram was developed in n-butanol:acetic acid:water (65:13:22, v/v) and visualized with the Van Urk reagent.

**Table II. TLC of Soybean Seed IAA Peptides**

| Substance | Rf  
|-----------|-----
| IIAasp    | 0.25 0.17 0.33  
| Compound 2| 0.25 0.18 0.33  
| IAGlu     | 0.36 0.22 0.36  
| Compound 3| 0.38 0.22 0.36  

Compounds 2 and 3 both yielded IAA upon hydrolysis, although tryptophan was also present in the hydrolysates, apparently as a result of contamination of the two substances with compounds 1 and 4, respectively (Fig. 2). Fractionation of the PVPP- and ether-purified sample by HPLC as described by Hollenberg et al. (15) resulted in compounds 2 and 3 eluting with retention times identical to those of IIAasp and IAGlu (26.2 and 28.1 min), respectively. After this purification, compound 2 co-chromatographed with IAAsp, and compound 3 co-chromatographed with IAGlu in silica gel TLC in three solvent systems (Table II).

Additional confirmation of the identities of the two soybean IAA peptides was obtained with a sample purified using [14C] IAAsp and [14C]IAGlu as tracers. Radioactive HPLC fractions eluting at the retention times of IAAsp and IAGlu were pooled, methylated, and analyzed by GLC and GC-MS. Figure 3 shows the gas chromatograms of authentic bis-methyl-IAAsp and bis-methyl-IAGlu and of the methylated soybean sample. The glutamate derivative always chromatographed as two peaks with variable relative areas. GC-MS analysis of the second, major peak was consistent with the compound being bis-methyl-IAGlu (Fig. 4A). Bis-ethyl-IAGlu, synthesized directly from IAA and bis-ethyl-L-glutamate, also chromatographed as two peaks, indicating that the degradation of the compound took place during chromatography rather than in the esterification reaction. The chromatogram of the methylated soybean sample shows peaks coinciding with those obtained with the authentic peptide methyl esters, and these identifications were upheld by GC-MS analysis. Figure 4B shows a mass spectrum of the material eluting at the retention time of the second bis-methyl-IAGlu peak.
The more acids, the evidence peaks and soylbean-derived bis-methyl-IAAsp (B) have been shown to contain the two peptides as naturally occurring substances.

In studies of 2,4-D metabolism, Feung et al. (12) showed that soybean plants and tissue cultures were able to conjugate the synthetic auxin to alanine, valine, phenylalanine, tryptophan, and leucine, in addition to aspartate and glutamate. Although tissue differences between seeds and vegetative structures cannot be ruled out at this point, it is possible that IAA and 2,4-D may be metabolized by different pathways in soybeans. A difference between the two auxins in conjugation reactions has also been suggested by Michaleczuk and Bandurski (17) who reported that the corn kernel IAA-glucose synthase, catalyzing the first reaction in IAA- myo-inositol biosynthesis, can not use 2,4-D as a substrate.

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