An Improved Method for Analysis of Polyamines in Plant Tissue by Precolumn Derivatization with o-Phthalaldehyde and Separation by High Performance Liquid Chromatography

James L. Corbin, Barry H. Marsh*, Gerald A. Peters*2
Battelle-Kettering Research Laboratory, Yellow Springs, Ohio 45387

ABSTRACT

An improved high-performance liquid-chromatographic method was developed for estimation of polyamines in crude plant extracts. Polyamines were derivatized with o-phthalaldehyde and mercaptoethanol (OPT). The fluorescent derivatives were eluted from a C18 column with the dimethylcyclohexylamine-phosphate buffer derived by T. Skauen and T. Greibrokk ([1982] J Chromatogr 247: 111-122) after treatment to remove impurities in the buffer. The method had a sensitivity of 1-2 picomoles and completely resolved nine polyamines (agmatine, spermine, nor-spermidine, spermidine, 3,5-homospermidine, 4,4-homospermidine, 1,3-diaminopropane, putrescine, and cadaverine) in 12 to 14 minutes. An optional ion-exchange step was used to remove less basic amines (including amino acids) and to concentrate the crude extracts. This method was compared with benzoyl chloride derivatization. Use of the benzoyl chloride method vastly underestimated the amount of polyamine in some plant extracts, a problem not encountered with the OPT procedure. Additionally, the OPT procedure resolved two isomers of homospermidine found in Azolla caroliniana. These two isomers were not resolved with the benzoylation method. Overall, the OPT method described here requires preparation and analysis time similar to other current methods but provides greater sensitivity and selectivity.

The PAs4 Put, Spd, and Spm occur widely in microorganisms, animals, and plants (17). While required for normal growth and development, their precise functions are unknown. PA synthesis inhibitors are known to arrest development or differentiation in invertebrates, birds, mammals (4, 7), and plant cell cultures (2,10). The role of PAs in nucleic acid and protein synthesis is well documented, and they may also have a role in plant senescence (16). Current interest in PAs is such that the capability to identify and quantify these compounds is desirable in laboratories not routinely involved in their analysis. Therefore, we examined existing methods for PA analysis taking into consideration their sensitivity, rapidity, whether or not they required dedicated instrumentation, and their general applicability.

The structure of PAs does not allow for low level optical detection, and thus most analyses rely on derivatization of the sample before detection. Perhaps the most widely used procedure involves dansylation followed by TLC separation and spectrophotometric quantification (3, 13). Analysis by HPLC using prechromatographic derivatization with dansyl chloride or other reagents such as benzoyl chloride, fluorescamine, and OPA plus mercaptoethanol have also been reported (14). Acid chlorides such as dansyl chloride and benzoyl chloride react with cell constituents such as phenolic hydroxyls, imidazole nitrogen, and some alcohols in addition to the intended amines (14). HPLC techniques such as ion-exchange chromatography or reversed-phase ion-pair chromatography offer the advantage of potential automation, but ion-exchange chromatography can suffer from long analysis times, and reversed-phase ion-pair chromatography may not resolve Dap from Put (14). Procedures such as these use postcolumn derivatization and therefore require an additional pump for the derivatization reagent.

From these considerations, prechromatographic derivatization with OPT followed by reverse-phase HPLC and fluorometric detection seemed to offer advantages over other current techniques. This method is more sensitive than benzoylation and UV detection (15) and more selective in that it reacts only with primary amines. In this study, we modified a recent procedure (15) to extend the range of compounds resolved and to reduce the analysis time. We also added a buffer purification step, which reduced background impurity peaks and allowed increased resolution of the component peaks. An additional sample clean-up step provided greater selectivity without increasing sample preparation time beyond that of other current methods. PA levels determined in a variety of plant material by the OPT and the benzoyl chloride methods are presented and compared.

MATERIALS AND METHODS

Plant Material

The symbiotic N2-fixing association Azolla-Anabaena was cultured as described previously by Peters et al. (12). Oat...
Table I. Comparison of the Separation of Benzoyl-Polyamines and OPT-Polyamines

<table>
<thead>
<tr>
<th>Benzoyl-Polyamines</th>
<th>OPT-Polyamines</th>
</tr>
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<tbody>
<tr>
<td>Polyamine</td>
<td>Retention time</td>
</tr>
<tr>
<td>Put</td>
<td>4.39 ± .07</td>
</tr>
<tr>
<td>Dap</td>
<td>4.65 ± .07</td>
</tr>
<tr>
<td>Cad</td>
<td>4.96 ± .07</td>
</tr>
<tr>
<td>Agm</td>
<td>5.66 ± .13</td>
</tr>
<tr>
<td>n-Spd</td>
<td>6.52 ± .07</td>
</tr>
<tr>
<td>Spd</td>
<td>7.53 ± .08</td>
</tr>
<tr>
<td>4,4-Spd</td>
<td>8.67 ± .24</td>
</tr>
<tr>
<td>3,5-Spd</td>
<td>8.67 ± .24</td>
</tr>
<tr>
<td>Spm</td>
<td>13.12 ± .24</td>
</tr>
</tbody>
</table>

Table II. Polyamine Levels in Azolla Frond, Pea Internode, Oat Leaf, Soybean Leaf, and Alfalfa Sprout Tissue

An aliquot of each tissue extract was derivatized with benzoyl chloride (Benzoyl) and with OPT. The benzoyl-polyamines were eluted isocratically from a C18 column in 60% (v/v) methanol while the OPT-polyamines were eluted from a C18 column with a gradient composed of phosphate-DMCA buffer in 50% (v/v) methanol. Values are the mean ± standard deviation of triplicate samples. Components not detected are indicated by ND.

(Avena sativa) and pea (Pisum sativum cv Alaska) were grown in a mixture of Turface (Montmorillonite clay, International Mineral and Chemical Corp., Mundelein, IL) and potting soil at 26°C and constant light of 280 μmol mol m⁻² s⁻¹ provided by a mixture of fluorescent and incandescent light in a controlled environment chamber. Soybeans (Glycine max cv Williams) were grown in Turface under a 16-h photoperiod in a growth chamber at a photosynthetic flux density of 260 μmol mol m⁻² s⁻¹ also provided by a mixture of fluorescent and incandescent bulbs at a temperature of 25°C day and 22°C night. Fresh alfalfa sprouts were purchased from a local market.

Chemicals

2-Mercaptoethanol (stored at -10°C under argon in a crimp-top vial), Put, Agm, Spd, and Spm were purchased from Sigma Chemical Co. Cadaverine, n-Spd, and Dap were
A, Benzoyl-PA eluted isocratically with 60% (v/v) methanol-water at 1.6 mL/min. The peak labeled with an asterisk (*) has the same retention time as n-Spd (Table I). B, OPT-PA eluted with a gradient of methanol and phosphate-DCMA buffer in 50% (v/v) methanol at 1.6 mL/min. A peak with the same RT as n-Spd is not detected (Table I).

from Aldrich Chemical Co. The OPA was obtained as Fluor-aldehyde from Pierce Chemical Co and stored at -10°C. The DCMA (>99% pure) was purchased from Fluka AG. The 4,4 h-Spd was prepared by one of us (J. L. C.) according to Okada et al. (11). The 3,5 isomer (9) was made by applying the same approach used in (11) for the 4,4 isomer. All other chemicals and solvents were reagent grade. Polyamine standards, stored in polypropylene tubes as 10 and 1 mM aqueous stocks or as a 0.1 mM standard mixture at -10°C, were stable for several months.

Extraction

For benzoylation analysis, 100 to 150 mg fresh weight of tissue was ground with 1 mL of cold 5% (v/v) PCA in a motor-driven, Teflon-pestle homogenizer. For analysis by OPT, 100 to 300 mg fresh weight and 2 mL of PCA was used. For both analyses, the homogenate was poured into 15-mL Corex tubes on ice and then pelleted by centrifugation at 20,000g for 30 min in an SS34 rotor at 4°C. The clear supernatant was analyzed for soluble or ‘free’ PA.

HPLC

A Waters system consisting of a model 6000A and 510 pumps, U6K injector, and a model 680 gradient controller was used. Detectors included a model 440 fixed wavelength (254 nm) absorbance detector or a model 481 variable wavelength absorbance detector and a fluorescence detector (338/400 nm excitation/emission filters). A silica presaturation column (Whatman Solvecon, 37-50 μm, 4.6 mm × 25 cm) was used between the pump and injector.

Benzyol Chloride Derivatization and HPLC Analysis

Plant extracts and standards, benzoylated as described by Flores and Galston (3), were chromatographed on one of two
analytical columns: an Altex Ultrasphere-ODS (5 μm, 4.6 mm × 15 cm) with a Waters C18 guard module, or a Waters μBondapak C18 (10 μm, 3.9 mm × 30 cm) with a Brownlee Labs guard column (RP-18, OD-GU or NewGuard). There were no differences in performance, but we preferred the latter arrangement as it was free from periodic pressure increases that necessitated backflushing the analytical column. The benzoyl-PA were eluted isocratically at room temperature with 60% (v/v) methanol:water at a flow rate of 1.6 mL/min.

**OPT Derivatization and HPLC Analysis**

Extracts were treated by an ion-exchange procedure (8) before derivatization with OPT. Sample extracts (1 or 2 mL) were applied to a 0.7 mm × 2.3 cm column of Bio-Rad AG 50W-X4 cation exchange resin (H+ form, 200–400 mesh) in Kontes Econoflex 10 cm columns. The following elution volumes were experimentally determined to yield 90% recovery of Dap and 100% recovery of all other PA standards: 2.8 mL of 0.7 M NaCl in 0.1 M sodium phosphate (pH 8.0), 1.2 mL of water, 3.0 mL of 1 N HCl, and 2.4 mL of 6 N HCl. The 6 N HCl eluate was taken to dryness (rotary vacuum-evaporated, 40°C) and then dissolved in 200 μL of water for a 5- to 10-fold concentration of the original extract.

Derivatization with OPT reagent and HPLC analysis was modified from the report of Skaaden and Greibrokk (15). The OPT reagent was prepared by dissolving 43 mg of OPA and 32 μL of 2-mercaptoethanol in 0.8 mL of methanol and diluting this solution to 8 mL with 0.4 N sodium borate buffer (pH 10.8). The reagent, stored under argon in a crimp-cap vial at room temperature, was generally allowed to stand overnight to reduce the background fluorescence before its initial use. Stored in this manner the reagent was stable for several weeks. The sample (50 μL) was mixed with reagent (50 μL) and reacted for 90 s at room temperature before injecting 25 μL onto the HPLC column. For the PA standards, 25 μL of the standard stock and 75 μL of the reagent were used.

The derivatized PAs, chromatographed on an Altex Ultrasphere-ODS (5 μm, 4.6 mm × 15 cm) analytical column with a C18 Brownlee Labs guard column (NewGuard), were eluted with methanol (solvent A) and 0.1 M phosphate (pH 4.0) plus 0.05 M DCMA in 50% (v/v) methanol:water (solvent B, see below) at a flow rate of 1.6 mL/min according to the following gradient program: 0 to 2 min 20% A, 80% B; 2 to 10 min linear gradient to 80% A, 20% B with a 2.3 min hold; 12.3 to 12.5 min linear ramp to initial conditions. Samples could be injected every 20 min. A Hewlett-Packard model 3390 A recording integrator was used to chart the fluorometer output.
and quantify the results based on comparison with RT of authentic compounds. In some experiments, Cad or the 3,5 isomer of h-Spd was used as an internal standard.

Buffer Purification and Preparation of Solvent B

The buffer for solvent B was made by combining one-half mol each DCMA and NaOH in about 900 mL water with enough phosphoric acid to bring the pH to 7.3. A solution of OPA (300 mg) and i-butylmercaptan (200 μL) in 50 mL of 95% (v/v) ethanol was added to the buffer and stirred overnight under argon before being transferred to a separatory funnel and extracted four times with 175-mL portions of ethyl acetate and once with 175 mL of pentane to remove OPT-reactive impurities (1). The solution was adjusted to pH 4.0 with concentrated phosphoric acid and the residual pentane removed by boiling gently for a few minutes. The hot buffer was then treated with 2 to 3 tablespoons of activated charcoal, cooled, and filtered through a 0.2 μm filter. The buffer was readjusted to pH 4.0 with phosphoric acid if necessary. Based on the total amount of phosphoric acid used, the volume was adjusted with water to provide 1 mL phosphate. Solvent B was prepared by adding 100 mL of this purified buffer to 500 mL of methanol and diluting to 1 L with water.

RESULTS AND DISCUSSION

The elution pattern of the benzoyl-PA shown in Table I differs from previously published work (3) in that Put elutes before Dap. This invariably occurred in our hands even though columns from the same manufacturer were used in our study and in the previous work. The reason for the discrepancy is not known. The RTs were reproducible within ±1 to 3% for Spd or h-Spd, respectively. Detection limits were comparable to those reported (3), though we found that detection at 245 nm gave an almost threefold increase in sensitivity compared to detection at 254 nm. With the exception of incomplete resolution of Dap from Put and Cad, all components were resolved and eluted in less than 15 min. Biological samples frequently contained one, late-eluting peak of unknown origin that interfered with early peaks of the subsequent run. The time between successive analyses was about 18 min. Though Dap was not completely resolved from Put and Cad with this procedure, few plant species examined to date have Dap in appreciable quantities (Table II; 3). Therefore, Put was resolved from Cad in plant samples without Dap.

Of more concern to us was the fact that the benzoylation method could not resolve the two isomers of h-Spd (Table I). The PAs of the Azolla-Anabaena association were an area of specific interest. The 3,5-isomer has been reported as the major PA of N₂-fixing cyanobacteria (6), and we wanted a procedure that would allow us to distinguish the isomers. Therefore, we explored derivatization with an OPT reagent and fluorescence detection (15) and subsequently made several modifications to the referenced method that increased resolution and sensitivity.

Increasing the pH of the elution buffer from pH 2.8 to 4.0 (before methanol addition) increased the response of Dap significantly without altering the elution profile (data not shown). With gradient elution, RT varied less than 1% for Put and 3% for Agm (Table I). All components, including the two isomers of h-Spd, were resolved in less than 15 min. The practical detection limit was 1 to 2 pmol, though a 0.5 pmol limit has been reported (15). Previous workers using the phosphate-DCMA buffer observed an interfering peak attributed to an impurity in the derivatization reagent which eluted between Spm and Spd (15). We determined that the interfering peak was due to an impurity in the elution buffer, not the derivatization reagent, and that it could be removed by a purification scheme devised earlier to purify buffer for a rapid HPLC ammonia determination (1). Removal of the impurity peak not only enhanced the resolution of adjacent peaks, but also allowed the gradient to be restructured, reducing analysis time.

Compared to benzoyl chloride derivatization, the OPT derivatization method offers complete resolution of a more complex component mixture and more selectivity in a comparable analysis time. OPT specifically reacts only with primary amines under the derivatization conditions, while acid chlorides such as benzoyl chloride and dansyl chloride can react with numerous components in plant extracts other than amines to yield UV-absorbing derivatives (3, 14). Chromatograms obtained after reaction with benzoyl chloride can also be further complicated by a wide variety of plant constituents that absorb at 245 nm or at 254 nm. For example, a chromatogram of benzoyl-PA from an extract of Azolla showed a UV-absorbing peak that eluted with the same retention time as n-Spd (Fig. 1A). The same extract derivatized with OPT showed fluorescent peaks with the same RTs as all PAs found in the benzoylated extract except n-Spd (Fig. 1B). In addition to coelution with authentic compounds, the use of the OPT reaction can help establish peak identity by verifying that the component is indeed a primary amine.

The crude plant extracts were routinely subjected to purification by ion-exchange. This step not only separates the strongly basic PAs from weaker bases, including OPT-reactive amino acids, but also gets rid of acidic and neutral compounds which could contaminate the HPLC column or interfere with the analysis (Fig. 2). Even basic amino acids such as arginine, ornithine, and lysine are eliminated from the PA fraction with this ion-exchange step (Fig. 3). This purification step is highly recommended and serves as further assurance that only strong base primary amines (such as PA) are being analyzed. Sample preparation time for the OPT/ion-exchange procedure took no longer than for the benzoyl chloride derivatization.

The soluble PA contents in extracts from several plants determined by benzoyl chloride derivatization and by OPT derivatization are compared in Table II. The two methods agree well for Azolla frond tissue, but in other plant tissues the levels obtained with benzoyl chloride were significantly lower than those determined by the OPT method. With the exception of soybean leaf tissue, the results obtained with the two methods are more similar when the data are expressed as percent of total (Table II).

The qualitatively similar but quantitatively different results led us to examine whether or not the benzoylation reaction might be inhibited in the presence of the crude extracts. This
was tested in two ways. First, a soybean leaf extract was spiked with a set of PA standards prior to derivatization with benzoyl chloride and the percent recovery of the standards determined. The recoveries ranged from 48% (Spd) to 64% (Spd).

In the second approach, extracts of Azolla fronds and of soybean leaf were spiked with Cad as an internal standard. An aliquot of the extract was then benzoylated directly or put through the ion-exchange purification step before benzoylation. As a control, an aliquot of PCA with Cad was treated in the same manner as the samples. The area of this internal standard peak was then compared in the two treatments. Additionally, authentic dibenzoyl-Cad (synthesized by J. L. C.) was added to the crude extract and then the extract was benzoylated. Comparing the control value with the value of dibenzoyl-Cad presented in Table III, it appears that 100% of the Cad added to the PCA in the control is derivatized by this procedure. In the presence of the Azolla and soybean leaf crude extracts, 76 and 3%, respectively, of the added Cad is recovered in a compound with the same retention time as authentic dibenzoyl-Cad. It is also clear from the results that significantly more of the Cad added to the extracts is recovered as putative dibenzoyl-Cad if the extracts are first purified by passage through an ion-exchange column and then benzoylated. After purification, 95% of the Cad added to the Azolla extract is recovered as putative dibenzoyl-Cad. For the soybean leaf extract, this figure is 83%, compared with just 3% if the ion-exchange step is not used. One interpretation of these results is that there is a compound(s) in the crude extract which inhibits the benzoylation reaction and that this compound is at least partially removed by the ion-exchange step. These results suggest that the ion-exchange purification step may be worthwhile even in connection with the benzoyl chloride derivatization procedure.

The sample preparation and analysis time for the OPT/ ion-exchange method described here is comparable to other current methods of PA analysis but has important advantages of specificity, sensitivity, and selectivity. Though it does not lend itself to automation due to the decay of the fluorescent product, it is a generally applicable method that should be beneficial to laboratories interested in PA analysis of crude plant extracts.

LITERATURE CITED


