Identification of a Dihydrophaseic Acid Aldopyranoside from Soybean Tissue

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ABSTRACT

A previously unidentified abscisic acid metabolite has been isolated and characterized. \((\pm)-[2-1^{14}C]\)Abscisic acid was incubated in intact soybean leaves and pods; the radiolabeled metabolite was purified by high performance liquid chromatography with on-line scintillation spectrometry detection. Gas chromatography-mass spectrometry was used to obtain spectra of the acetylated and methyl esterified derivatives. The data were consistent with a proposed dihydrophaseic acid-aldopyranoside identity. Conjugation through the 4'-hydroxyl of dihydrophaseic acid is suggested.

The metabolism of AbA appears to be an important endogenous mechanism by which this biologically active compound is removed from plant tissue (7, 13). The main pathway of AbA metabolism is hydroxylation and rearrangement to PA and the subsequent reduction to DPA (7, 13). In recent studies with \((\pm)-[2-1^{14}C]\)AbA introduced into soybean leaves and pods, a major proportion of the radiolabel was associated with an unidentified metabolite (11). This metabolite was more polar than DPA and accumulated as \(^{14}C\) incorporation into DPA approached a peak. The compound was not alkali-labile and could be methylated with diazomethane, suggesting the presence of a free carboxyl group. An AbA metabolite with similar characteristics has recently been found in barley (3). We now report the postulated identity of this compound based on the mass spectra of its acetate and methyl ester derivatives.

MATERIALS AND METHODS

Pods on intact soybean (Glycine max [L.] Merr., cv. 'Clay') plants, which were in the late-pod filling growth stage, were injected with 20 \(\mu\)g/seed of 50 mg/l \((\pm)-[2-1^{14}C]\)AbA (1.2 \(\mu\)Ci/mol) in 95% (v/v) ethanol/water. After 2.5 days the pods were harvested, extracted in 80% (v/v) methanol/water, and dried in vacuo as described elsewhere (10).

AbA metabolites in crude extracts were separated by reverse phase HPLC on a 250 \(\times\) 10 mm (i.d.) column (R Sil, \(\mu\)C18, 10 \(\mu\)m particle size; Alltech, Arlington Heights, IL). Samples were dissolved in 3.5 ml aqueous 0.1 n acetic acid, filtered through a 5-\(\mu\)m pore-size filter (LC-type, Millipore Corp.), injected onto the column in 5 ml, and eluted at 3 ml/min with a solvent gradient linearly changed over 30 min from 0.1 n acetic acid in water to 0.1 n acetic acid in 48% (v/v) ethanol/water. The presumed DPA aldopyranoside was collected at a retention time of 18.7 min. As previously reported (11), DPA eluted at 24.1 min and AbA at 33.5 min with this system.

Radioactivity for all HPLC procedures was monitored with an on-line scintillation counter (model 1050, CAI Instruments, Inc., Midland, MI) and a 100 \(\times\) 4 mm (i.d.) quartz detector cell (model 3-FL) packed with glass-encapsulated scintillant (model EGB-02, CAI Instrument, Inc.).

Further purification was accomplished by HPLC on a 150 \(\times\) 4 mm (i.d.) column (\(\mu\)Bondapak NH2, 10 \(\mu\)m spherical particle size, Waters Assoc., Milford, MA). The samples were injected in 1 ml solvent consisting of 0.1 n acetic acid in acetonitrile:ethanol:water (70:29:1, v/v) and eluted at 1 ml/min with a second order concave solvent gradient (program no. 8, model 660 Programmer, Waters Assoc.) changed to 0.1 n acetic acid in 95% (v/v) ethanol/water in 30 min. The presumed DPA aldopyranoside eluted at 10.2 min with this system.

Acetylation was accomplished by suspending 0.1-mg samples in 100 \(\mu\)l dry pyridine and 100 \(\mu\)l acetic anhydride and incubating at room temperature for 25 h. Samples were dried in vacuo at 40 C and then further purified by reverse phase HPLC on an analytical-sized 130 \(\times\) 4 mm (i.d.) column (\(\mu\)C18 Bondapak, 10 \(\mu\)m spherical particle size, Waters Assoc., Milford, MA). Samples were injected in 1 ml aqueous 0.1 n acetic acid and eluted at 1 ml/min with a first order convex solvent gradient (program no. 5, model 660 Programmer, Waters Assoc.) changed from aqueous 0.1 n acetic acid to 0.1 n acetic acid in 29% (v/v) ethanol/water in 20 min. The presumed acetyl DPA aldopyranoside was collected at 9.4 min. The unacetylated compound eluted at 11.3 min on this system. Methylation was with diazomethane as described by Schlenk and Gellerman (9).

Gas Chromatography-Mass Spectrometry. Mass spectra were obtained using an LKB 9000 gas chromatography mass spectrometer interfaced to a PDP 8e computer under spectrometric conditions as follows: ion source 290 C, separator 280 C, and ionization potential of 20 ev. The samples were run on a 60 cm \(\times\) 2 mm (i.d.) glass column packed with 3% OV-1 on 100 to 120 mesh Supelcoport with temperature programmed from 220 to 290 C at 10 C/min with a helium flow rate of 25 ml/min.

RESULTS AND DISCUSSION

Previously, we found (11) that the predominant metabolites of exogenously introduced \(^{11}C\)AbA formed after 1 to 2 days of incubation were PA, DPA, presumed AbA pyranose ester, and a
FIG. 1. Mass spectrum of presumed DPA aldopyranoside tetracetate. Relative intensity is normalized to m/z 331 rather than the base peak of 169. Relative intensities of peaks at m/z 109, 169, and 264 were 2,400, 2,900, and 1,000%, respectively.

FIG. 2. Mass spectrum of presumed methyl ester of DPA aldopyranoside tetracetate. Relative intensity is normalized to m/z 331 rather than the base peak of 169. Relative intensities of m/z 109 and 169 were 120 and 310%, respectively.

previously unidentified compound which we now propose is a DPA aldopyranoside.

The aldopyranose functional group is suggested by the peaks at m/z 331, 271, 211, 169, and 109 (2, 4, 8) for the acetate (Fig. 1) and the methyl acetate (Fig. 2) derivatives. Also consistent with the DPA pyranose are the apparent molecular ions for the underivatized compound (m/z 458, relative intensity 4%, data not shown), the presumed tetraacetate (m/z 612, Fig. 1), and the methyl ester of the presumed tetraacetate (m/z 626, Fig. 2). Fragments characteristic of DPA methyl ester are found in Figure 2 for the presumed methyl ester of DPA aldopyranoside as peaks at m/z 296, 278, 246, 220, 188, 154, 125, 122, 109, and 43 (12). In addition to the m/z 246 peak, a strong peak at m/z 247 was found in both the tetraacetate (Fig. 1) and the methyl ester of the tetraacetate (Fig. 2). This is consistent with the proposed reaction sequence shown in Figure 3 for the methyl ester. A similar sequence for the tetraacetate would involve the loss of -OH rather than -OCH3.

Evidence suggesting that the compound is not a sugar ester of DPA, analogous to the reported AbA glucose ester (5) includes our observations: (a) that the compound was stable (no shift in retention time on analytical HPLC) to mild hydrolysis conditions (aqueous NH4OH, pH 11, 60°C for 2 h), (b) that following diazomethane treatment we observed a shift in retention time on analytical HPLC from 11.3 to 15.6 min, and an increase in apparent molecular ion from m/z 612 (Fig. 1) to 626 (Fig. 2), and (c) that there was no peak at 441 from the hydroxyisopherone part of such a molecule, as has been reported by Koshimizu (3) and observed by us for the AbA pyranose ester.

The proposed structures (Figs. 1 and 2) show the glycosidic
edge of AbA metabolism. This knowledge allows further studies to determine whether the compound has biological activity and whether the metabolism to this compound plays a role in regulating the level of AbA in plant tissues.

**LITERATURE CITED**

1. **BELKE CJ, B SUTT, ML BRENNER 1980** Metabolism and phloem transport of [2-


7. **MIBORROW BU, G VAUGHAN 1979** The long term metabolism of (+)-[2-14C]-


**FIG. 3.** Proposed reaction sequence of presumed methyl ester of DPA tetraacetyaldopyranoside accounting for the observed ion fragments.

bond through the 4' carbon of DPA. This proposal is based on the reported difficulty of acetylation of the hydroxyl at C-1' (5, 6) and the observed molecular ions which are appropriate for a tetraacetate (Figs. 1 and 2).

The proposed DPA aldopyranoside was the most polar of the AbA metabolites observed after 2 days of incubation of [14C]AbA in soybean pods and leaves; and it accumulated as 14C incorporation into DPA reached a peak (11). This agrees with research with other plant species (1, 7, 12), indicating that as incubation time was increased, the metabolites which were formed had greater polarity.

The identification of DPA aldopyranoside extends our knowl-