Note

Stereoselectivity of Abscisic Acid-oxygenase in Avocado Fruits

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The optically resolved (±)-dihydrophaseic acid (DPA) was achieved by using a commercially available chiral HPLC column. PA and DPA, which were isolated after feeding (±)-(RS)-[2H₈]-ABA to avocado fruits, were analyzed by the chiral HPLC method to examine the stereoselectivity of the oxygenase. Small peaks of the unnatural enantiomer could be observed in each case. The results show convincingly that (−)-(R)-ABA was converted to PA and DPA, although the extent of this conversion is very small in comparison with the conversion of (±)-(S)-ABA.

The catabolism of (±)-(S)- and (−)-(R)-absic acid (ABA, 1 and 2) is distinctly different, (±)-(S)-ABA being metabolized rapidly via two major pathways. One involves conjugation with glucose to form an ABA-glucose ester, and the other involves oxidation of ABA by ABA-oxidase, the enzyme that forms 8'-hydroxy ABA from ABA, and conversion of this into phasic acid (PA, 3).1–8 This is further converted to dihydrophaseic acid (DPA, 4).9 The (R)-enantiomer is metabolized slowly to two conjugates, the glucose ester and 1'-glucoside, and to a minor acidic metabolite, but not to PA or DPA.5,7 Boyer and Zeervaart10 have suggested that ABA oxygenase in Xanthium leaves may hydroxylate the 7'-methyl of the unnatural (−)-(R)-isomer to give 7'-hydroxy-(−)-(R)-ABA (5). The formation of this compound has also been observed in Hordeum leaves11 and Vicia faba.12 However, these experiments did not establish the optical purity of the metabolites.

We have recently achieved the direct optical resolution of (±)-ABA and its metabolite, (±)-PA, by HPLC in a chiral stationary phase.13,14 In the present study, we achieved the chromatographic separation of (±)-DPA methyl ester and applied these results to examine the stereoselectivity of oxygenase activity. The stereoselectivity of the enzyme was investigated by chiral HPLC and EI-MS analyses of PA and DPA formed by avocado fruit slices after the administration of (±)-(RS)-[2H₈]-ABA.

Our previous studies indicated that an ovomucoid-conjugated column had no enantioselectivity for (±)-DPA, but that (±)-PA could be resolved.14 The direct enantiomeric resolution of (±)-DPA was reinvestigated by HPLC in a chiral stationary phase. The methyl ester of (±)-DPA could only be resolved in a cellulose tris(3,5-dimethyl phenylcarbamate) column (Chiralcel OD, system A), the retention times of the natural enantiomers always being shorter than those of the unnatural enantiomers. This is the first example of the optical resolution of (±)-DPA methyl ester.

We fed unresolved racemic [2H₈]-ABA to avocado fruits instead of resolved (±)-(S)- and (−)-(R)-[2H₈]-ABA so that we could examine the metabolism of each enantiomer under the same conditions at the same time. The tissue was extracted, and biosynthesized PA and DPA were assayed and purified by HPLC. The incorporation of the label was measured by EI-MS. The mass spectra of the PA and DPA methyl esters after administering (±)-(RS)-[2H₈]-ABA indicated ions 4, 5, and 6 amu heavier than each molecular ion. The presence of these ions showed that 3H-labelled ABA was converted by an oxygenase into PA and DPA. The 3H-incorporation was measured by monitoring the ions between m/z 294 and 300 for the PA methyl ester and between m/z 296 and 302 for the DPA methyl ester. The 3H-enrichment of the isolated methyl ester of PA and DPA was 92 and 32%, respectively. Neither epi-PA nor 7'-hydroxy ABA was detected in the extract of avocado by GC-FID and GC-SIM. Isolated PA and DPA were analysed by stereochemical analytical methods to evaluate the optical purity of each. If only the unnatural enantiomer of ABA could be metabolized by an oxygenase, no peak from the unnatural form would be observable in the chromatograms. Chiral HPLC (system B) showed that the labelled PA was a mixture containing 6.5% of the unnatural enantiomer and 93.5% of natural PA. In the case of DPA, a small peak was eluted at the same retention time as that of the unnatural enantiomer of the DPA methyl ester by chiral HPLC (system A), this comprising 1.6% of the unnatural enantiomer of the DPA methyl ester. These results indicate that (−)-(R)-ABA was also metabolized to a small extent by ABA oxygenase. From the deuterium content of methyl ester of PA and DPA calculated by EI-MS, and the peak area ratio of the unnatural form of PA and DPA, the optical purity of PA and DPA was found to be 86.0 and 90.4% e.e., respectively. Although racemase activity has not been ruled out, its presence is considered unlikely because no unnatural form of PA and DPA could be detected in the PA and DPA isolated from the control experiment. From these results the conversions were confirmed to be of ABA oxygenase.

Some workers have already observed that tomato shoots could metabolize (−)-(R)-ABA into PA and DPA.5,15 The products have been thought to be formed by minor contamination of the (−)-(S)-enantiomer in (−)-(R)-ABA. Dashek et al. have also

Fig. The Chemical Structure of Each Compound is Shown as an optically Active form

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reported that barley aleurone layers could convert unnatural enantiomer of ABA into PA and DPA.\textsuperscript{16} Our present experiments support these observations and that indicate these products were formed from the (−)-(R)-enantiomer by ABA oxygenase. Thus, ABA oxygenase is not fully specific for natural (+)-(S)ABA. We obtained no evidence that this enzyme was involved in the formation of 7'-hydroxy ABA.

**Experimental**

**Chemicals.** (1'S,2'R,6'R)-PA (natural form, 3) and (1'R, 2'S, 6'S)-PA (unnatural form) were supplied by Dr. T. Kitahara. (1'S,2'R,4'S,6'R)-DPA (natural form, 4) and was prepared by reducing\textsuperscript{3,17} while (1'R,2'S,4'R,6'S)-DPA (unnatural form) was prepared by reducing the unnatural form of PA.\textsuperscript{17} (1'S,2'S,6'S)-epi-PA was provided by Profesor K. Yamashita as the methyl ester.

**Labelled compound.** (±)-(RS)-[\textsuperscript{2}H\textsubscript{6}]ABA was prepared by exchange in NaOD in D\textsubscript{2}O.\textsuperscript{18}

**Plants.** Avocado fruit (Persea americana Mill.) was purchased from a local supplier.

**Administration of the labelled compound to the plant.** Avocado fruits were kept until they began to soften. Then cut in half and the seeds removed. Longitudinal and transverse cuts (a total of about 450 g of fruit) 30-40 mm apart were taken and on the newly exposed surface of each slice was applied 50-100 µl (Triton X-100: Me\textsubscript{2}CO:H\textsubscript{2}O; 1:1:8, by vol.) of \textsuperscript{1}H-labelled compound solution. The treated slices were placed in a water-saturated atmosphere and covered with a plastic bag for 24 h.

**Extraction.** MeOH extracts of the avocado fruits were concentrated and extracted with n-hexane. The aq. layer was acified and extracted with EtOAc, and the EtOAc-soluble fraction was chromatographed in silica gel column with a mixture of EtOAc and toluene containing 1% HOAc. ABA was eluted with 40% EtOAc, PA was eluted with 50% EtOAc, and DPA was eluted with 100% EtOAc. Each fraction was further purified for the EI-MS and the chiral HPLC analyses by preparative HPLC in a Sumipax ODS A-212 column (6.0 x 150 mm), eluting with 50% MeOH containing 0.1% HOAc at 1.0 mL/min: PA (300 µg) \textsubscript{t} 6.0 min, 5.5-6.5 min collected; DPA (1 mg) \textsubscript{t} 4.7 min, 4.2-5.2 min collected. The purities of PA and DPA were checked by rechromatography in the same column, no impurities were observed in either case. Aliquots of each were then analysed for their \textsuperscript{3}H content by EI-MS after methylation.

**Mass spectroscopy.** EI spectra of Me-PA and Me-DPA were obtained to determine their \textsuperscript{3}H content with a HITACHI M-80B instrument interfaced with a HITACHI M-0101 data system, using an electron energy of 70 eV and probe temp. of 180°C. The \textsuperscript{3}H contents were calculated after correction for isotopic contributions.

**Assay of epi-PA and 7'-hydroxy ABA.** A sample of about 450 g of avocado fruits after being administered with (±)-(RS)-[\textsuperscript{2}H\textsubscript{6}]ABA was subjected to the extraction and purification methods outlined in the previous section. The fractions eluted between with 5% and 100% EtOAc (a total of 9 fractions) were analysed by GC-FID (2% OV-17, system C) and GC-SIM (DB-1, system D) after methylation. When the GC-SIM (EI) analysis for 7'-hydroxy ABA-Me was done, the ions at \textit{m}/\textit{z} 238 [M–C\textsubscript{6}H\textsubscript{5}]\textsuperscript{+}, 206 [M–C\textsubscript{6}H\textsubscript{5}–MeOH]\textsuperscript{+}, 188 [M–C\textsubscript{6}H\textsubscript{5}–MeOH–H\textsubscript{2}O]\textsuperscript{+}, and 125 (ABA-like side chain) were monitored.\textsuperscript{19}

**Chromatography.** HPLC systems: A. Chiraicel OD (Daicel, Tokyo, Japan, 250 x 4.6 mm i.d.) eluted isocratically with n-hexane-2-ProH (9:1, v/v) at 1 mL/min\textsuperscript{−1}, eluate monitored at 254 nm; the (±)-DPA methyl ester was eluted at 7.6 and 9.4 min, respectively. B. ULTRON ES-OVM (Shinwakko, Kyoto, Japan, 150 x 4.6 mm i.d.) eluted isocratically with a 2-ProH-20 mK Pi-buffer (pH 3.5, 2:98, v/v) at 1 mL/min\textsuperscript{−1}, eluate monitored at 254 nm; (±)-PA was resolved by eluting the unnatural and natural isomers at 5.6 and 8.9 min, respectively.\textsuperscript{14}

**GC systems:** C. 2% OV-17 (1000 x 3 mm i.d.) at 210°C (isothermal), N\textsubscript{2} at 60 mL min\textsuperscript{−1}; epi-PA-Me was eluted at Rt 9.9 min under these conditions. D. DB-1 (3000 x 0.53 mm i.d., 1 µm film thickness) with a temperature programmed from 170 to 230°C at 5°C min\textsuperscript{−1}), He at 30 mL min\textsuperscript{−1}, linked to MS via single-stage jet separator at 240°C; MS resolution > 800; ionizing voltage, 20 eV; source, 180°C.

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**References**