Abscisic Aldehyde Is an Intermediate in the Enzymatic Conversion of Xanthoxin to Abscisic Acid in *Phaseolus vulgaris* L. Leaves

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**ABSTRACT**

The enzymatic conversion of xanthoxin to abscisic acid by cell-free extracts of *Phaseolus vulgaris* L. leaves has been found to be a two-step reaction catalyzed by two different enzymes. Xanthoxin was first converted to abscisic aldehyde followed by conversion of the latter to abscisic acid. The enzyme activity catalyzing the synthesis of abscisic aldehyde from xanthoxin (xanthoxin oxidase) was present in cell-free leaf extracts from both wild type and the abscisic acid-deficient molybdopterin cofactor mutant, Az34 (nar2a) of *Hordeum vulgare* L. However, the enzyme activity catalyzing the synthesis of abscisic acid from abscisic aldehyde (abscisic aldehyde oxidase) was present only in extracts of the wild type and no activity could be detected in either the leaf or water stressed leaves of the Az34 mutant. Furthermore, the wilted tomato mutants, *sitlens* and *flaca*, which do not accumulate abscisic acid in response to water stress, have been shown to lack abscisic aldehyde oxidase activity. When this enzyme activity was isolated from leaf extracts of *P. vulgaris* L. and added to extracts prepared from *sitlens* and *flaca*, xanthoxin was converted to abscisic acid. Abscisic aldehyde oxidase has been purified about 145-fold from *P. vulgaris* L. leaves. It exhibited optimum catalytic activity at pH 7.25 in potassium phosphate buffer.

We have been working on the hypothesis that the biosynthesis of ABA in higher plants occurs via an indirect pathway involving the oxidative cleavage of a xanthophyll to a C15 compound which is subsequently metabolized to ABA. Although it has also been suggested that the synthesis of ABA in higher plants proceeds via the direct pathway involving a C15 precursor derived from farnesyl pyrophosphate (12), evidence has been accumulating to support the indirect pathway of its biosynthesis. Various corn mutants whose inability to accumulate ABA seems to be associated with carotenoid deficiency have been reported (4, 13, 14). Furthermore, inhibitors of carotenoid biosynthesis such as fluridone and norflurazon also inhibit the accumulation of ABA (6, 7). Creelman and Zeevaart (1) showed that leaves stressed in the presence of 18O2 incorporated one atom of 18O into the carboxyl group of ABA suggesting that a preformed xanthophyll was cleaved by a dioxygenase to form an aldehyde that was converted to ABA by dehydrogenases. Gage et al. (5) have reported that ABA synthesized in maize embryos under nonstress conditions also required a xanthophyll precursor. Based on 18O-labeling experiments, it has been suggested that ABA is derived, at least in part, from violaxanthin (9). Recently, Li and Walton (10) have found a 1:1 relationship on a molar basis between the reduction in levels of violaxanthin, 9'-cis-neoxanthin, and 9-cis-violaxanthin, and the accumulation of ABA, phaseic acid, and dihydropsehace acid in dark-grown water-stressed leaves of *Phaseolus vulgaris* L. Furthermore, leaves which had been detached but not stressed did not accumulate ABA and their xanthophyll levels were not reduced (10). Based on 18O-labeling patterns observed in ABA from different tissues, Zeevaart et al. (24) have recently concluded that, despite variations in precursor pool sizes and intermediate turnover rates, there is a universal pathway of ABA biosynthesis in higher plants which involves cleavage of a larger precursor molecule, presumably an oxygenated carotenoid. If the biosynthesis of ABA were to proceed via the indirect pathway, one would expect that either xanthoxin or abscisic aldehyde or both should be intermediates. Xanthoxin is found in plants and is converted to ABA when fed to tomato and bean shoots (2, 21). 13C-Labeled xanthoxin has been fed by Parry et al. (15) to wild-type tomato and to ABA-deficient mutants. They found a substantial incorporation of 13C into ABA by the wild-type and *notabilis* homozygotes, whereas both *sitlens* and *flaca* homozygotes showed very poor incorporation of label into ABA (15). We have reported earlier that cell-free extracts prepared from the leaves of *P. vulgaris* L., *Pisum sativum* L., *Zea mays* L., *Cucurbita maxima*, and *Vigna radiata* L. convert xanthoxin to ABA (17). Cell-free extracts prepared from the turdug and water-stressed leaves of wild-type tomato and the wilt mutant *notabilis* converted xanthoxin to ABA at similar rates while extracts from *sitlens* and *flaca* showed little or no activity (18). Similar results were obtained when abscisic aldehyde was used as the substrate, suggesting that abscisic aldehyde is the immediate precursor of ABA (18). None of the tomato extracts showed any significant activity with xanthoxin acid, xanthoxin alcohol or ABA-14-trans-diol as the substrates (18). We have now separated the xanthoxin oxidizing activities into two different enzyme activities, one catalyzing the synthesis of abscisic aldehyde from xanthoxin (xanthoxin oxidase) and the other that of ABA from abscisic aldehyde (abscisic alde-
hyde oxidase). Abscisic aldehyde oxidase has been partially purified and characterized.

**MATERIALS AND METHODS**

**Plant Material**

Seeds of *Phaseolus vulgaris* L. cv Blue Lake were purchased from W. Atlee Burpee and Company. Seeds of *Lycopersicon esculentum* Mill cv Ailsa Craig wild type, *notabilis*, *flaca*, and *sitiens* were a gift from Dr. R. Horgan, University College of Wales, Aberystwyth, UK, and those of barley wild-type genotypes, Steptoe, and the molybdopterin cofactor mutant (Az34) from Dr. R. L. Warner, Department of Agronomy and Soils, Pullman, WA. The seeds were surface sterilized with 10% Clorox solution for 30 min, washed in sterile H2O, and then imbibed in sterile H2O for 6 to 8 h. These were then planted in flats in a soil:vermiculite (1:1, v/v) mixture and grown in the greenhouse at 21°C. For tomato plants, 5-week-old seedlings were transferred to 20-cm pots containing a 1:1 (v/v) mixture of soil:vermiculite and were watered twice daily. For water-stress experiments, the leaves were detached and allowed to lose about 12% of their fresh weight. The leaves were then wrapped in aluminum foil and stored at ambient temperature in the laboratory in the dark.

**Preparation of Enzyme Extracts**

Bean and barley leaves were extracted with a mortar and pestle in 50 mM KPi* (pH 7.25–7.50) (5.1 mL buffer/1.7 g fresh weight leaf tissue). The extracting medium for tomato leaves was 0.2 mM KPi (pH 7.5) containing 7.5 mM DTT. The extracts were passed through four layers of cheese cloth and centrifuged at 12,000g for 20 min at 4°C. The supernatant was fractionated with acetone as described below and used for enzyme assays. For studies with tomato leaves, the supernatant fraction was used for enzyme assays.

**Acetone Precipitation**

The supernatant fraction was precipitated with acetone as described below. To 5 mL of the supernatant fraction, 20 mL of acetone (−20°C) were added slowly with constant stirring. It was then centrifuged at 27,000g for 1 to 2 min at 4°C. The resulting precipitate was flushed with air and extracted with 2.5 to 5 mL of 50 mM KPi (pH 7.25–7.50). The extracts were centrifuged at 12,000g for 20 min at 4°C and this fourfold acetone precipitate was used as the source of enzyme activities catalyzing the synthesis of ABA from xanthoxin.

For onefold acetone precipitation, 5 mL of the supernatant fraction was treated with 5 mL of acetone (−20°C) slowly and with constant stirring. This was immediately centrifuged at 27,000g for 1 to 2 min at 4°C. The precipitate thus obtained was flushed with air and extracted with 2.5 to 5 mL of 50 mM KPi (pH 7.25–7.50), centrifuged at 12,000g for 20 min at 4°C, and used as the source of abscisic acid oxidase activity.

To the supernatant fraction obtained after onefold acetone precipitation, 15 mL more acetone (−20°C) was then added slowly and with constant stirring. This was immediately centrifuged at 27,000g for 1 to 2 min at 4°C and designated as the threefold acetone precipitate. The precipitate was then extracted with 2.5 to 5 mL of 50 mM KPi (pH 7.25–7.50), centrifuged at 12,000g for 20 min at 4°C, and used as the source of xanthoxin oxidase activity.

**Enzyme Assays**

The assay tubes contained 0.5 to 0.65 mL 0.2 mM KPi (pH 7.25), 0.1 to 0.25 mM enzyme extract, various amounts of xanthoxin or abscisic aldehyde, and NAD or NADP as described in the text in a total volume of 0.85 mL. The tubes were incubated at 28°C for 1 h. For estimation of abscisic aldehyde oxidase activity, the reaction was stopped by adding 90 μL of concentrated HCl. [3H]ABA (12000 dpm, specific activity 10 Ci/mmol [23]) was then added and the tubes were chilled and centrifuged. ABA was extracted from the above supernatants with CHCl3 using a Pasteur pipette as a separatory funnel. The CHCl3 fractions containing ABA were then dried and methylated with diazomethane in ether (16). Further purification of the ABA methyl ester by HPLC and quantification by GC were as described earlier (18).

For the estimation of xanthoxin oxidase activity, the reaction was stopped by adding 2 mL of 1% NaHCO3, followed by the addition of 2 mL of ethyl acetate. The samples were further extracted twice with 2 mL each of ethyl acetate and the ethyl acetate extracts combined and dried. This was then taken up in 50% aqueous methanol, filtered, and processed by HPLC on an ODS (4.6 x 150 mm) column. Elution was carried out with a linear gradient of 50 to 100% methanol in H2O in 15 min at a flow rate of 0.8 mL/min. Abscisic aldehyde formed was quantified by comparing with authentic standards. Since labeled abscisic aldehyde was not available, the amount of abscisic aldehyde formed could not be corrected for the losses during handling and processing. Hence, in kinetic experiments, a coupled assay was carried out using the fourfold acetone precipitate and the amount of ABA formed was estimated as described earlier (17, 18).

Protein was determined with the Bio-Rad Protein assay (Bradford method) using bovine plasma albumin as the standard.

**Preparation of Xanthoxin and Abscisic Aldehyde**

Xanthoxin was prepared by zinc permanganate oxidation of violaxanthin and neoxanthin (20) with slight modifications as reported earlier (18). Abscisic aldehyde was prepared by chromium trioxide/pyridine oxidation of xanthoxin (20) and purified as detailed elsewhere (18).

**GC-MS**

The identities of all of the compounds made chemically or enzymatically were confirmed by GC-MS. Methyl esters of ABA were analyzed by GC-MS with a Finnegan 4000 GC/MS/DS system. The GC was performed on a SPB-1 fused silica capillary column (30 m x 0.25 mm i.d.) with a film thickness of 0.25 mm (Supelco, Bellefonte, PA) using helium as the carrier gas at a flow rate of 1 ml/min. After a 2-min

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3 Abbreviation: KPi, potassium phosphate.
hold at 50°C, the oven temperature was raised by 10°C/min. For xanthoxin and abscisic aldehyde, the conditions were the same except that the column was 8 m long. Mass spectrometry was by electron impact at 70 ev.

RESULTS

Fractionation of Xanthoxin Oxidizing Activity

The supernatant fraction of bean leaf extracts was fractionally precipitated with acetone to yield onefold, threefold, and fourfold acetone precipitates which were tested for their ability to convert xanthoxin and abscisic aldehyde to ABA. The results reported in Table I show that the fourfold acetone precipitated fraction converted 37.5% of xanthoxin to ABA. The onefold and threefold acetone precipitates converted no more than 6% of xanthoxin into ABA when assayed separately. However, the enzyme activity could be recovered when these fractions were assayed together suggesting that the enzyme activity was composed of at least two separate activities. However, the onefold acetone precipitate converted 64% of abscisic aldehyde to ABA, whereas it had no xanthoxin to ABA oxidizing activity (Table I).

Xanthoxin Oxidase Activity in Supernatant Fraction of Bean Leaf Extracts

In our earlier experiments, it was found that the rate of conversion of xanthoxin to ABA was 10 to 15% when the supernatant fraction of the bean leaf extracts was used as the enzyme source (17). This low conversion of the substrate into ABA was because of the presence of natural inhibitor(s) of the enzyme activity, which are heat stable and dialyzable (17). Results reported in Table II show that when the supernatant fraction from bean leaf extracts was assayed, 46.5% of xanthoxin was converted to abscisic aldehyde. No abscisic aldehyde was formed when NADP was left out of the assay system suggesting that the enzyme has an absolute requirement for NADP. Natural inhibitor(s) present in the bean leaf extracts inhibit only the activity of abscisic aldehyde oxidase since there was a 7.7-fold increase in the enzyme activity when the natural inhibitor(s) were removed by acetone precipitation. Furthermore, this enzyme does not seem to require NADP since even in the absence of this cofactor the enzyme activity was 68% compared to that obtained in the presence of NADP. In later experiments, when the acetone precipitated enzyme was dialyzed overnight against 20 mM KPi, pH 7.25 in the presence of 20% glycerol, the enzyme showed comparable activity in the absence or presence of NAD or NADP suggesting that this reaction does not require the presence of these cofactors. Furthermore, the enzyme did not catalyze the conversion of ABA, in the reverse direction, to abscisic aldehyde even in the presence of NADPH. When the threefold acetone precipitate was assayed, it converted only 13.5% of xanthoxin to abscisic aldehyde compared to that converted by the supernatant fraction of the bean leaf extract. One possible explanation for this low enzyme activity in the three-

<table>
<thead>
<tr>
<th>Fraction Assayed</th>
<th>ABA Formed with Substrate</th>
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<tbody>
<tr>
<td></td>
<td>Xanthoxin</td>
</tr>
<tr>
<td>Fourfold acetone precipitate</td>
<td>375</td>
</tr>
<tr>
<td>Onefold acetone precipitate</td>
<td>59</td>
</tr>
<tr>
<td>Threefold acetone precipitate</td>
<td>59</td>
</tr>
<tr>
<td>Onefold acetone precipitate + threefold acetone precipitate</td>
<td>417</td>
</tr>
</tbody>
</table>

Table II. Enzyme Activities in Supernatant Fraction and Acetone Precipitates of Bean Leaf Extracts

Assay contained 1 μg xanthoxin or abscisic aldehyde, 1.18 mM NADP, and supernatant fraction equivalent to 100 mg fresh weight of leaf tissue. Incubation was at 28°C for 1 h.

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Abscisic Aldehyde Formed with Xanthoxin as Substrate</th>
<th>ABA Formed with Abscisic Aldehyde as Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>Complete system</td>
<td>465a</td>
<td>78a</td>
</tr>
<tr>
<td>Minus substrate</td>
<td>NDb</td>
<td>24a</td>
</tr>
<tr>
<td>Minus enzyme</td>
<td>ND</td>
<td>16a</td>
</tr>
<tr>
<td>Minus NADP</td>
<td>ND</td>
<td>405c</td>
</tr>
<tr>
<td>Boiled enzyme</td>
<td>ND</td>
<td>22a</td>
</tr>
<tr>
<td>Onefold acetone precipitate</td>
<td>ND</td>
<td>599</td>
</tr>
<tr>
<td>Threefold acetone precipitate</td>
<td>63</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Supernatant fraction assayed.  b ND = not detected.  c Fourfold acetone precipitated enzyme assayed.
fold acetone precipitate is that either a cofactor of this enzyme is removed in the onefold acetone precipitate or abscisic aldehyde formed may be converted to some other product, such as abscisic alcohol as suggested by Duckham et al. (3).

**Enzyme Activities in Wild-Type Hordeum vulgare L. and AZ34 Mutant Leaves**

*Hordeum vulgare* L. molybdopterin cofactor mutant, AZ34, has low basal levels of ABA as well as a reduced capacity for producing ABA in response to water stress (22). Xanthoxin oxidase activity was present in the leaf extracts of both the wild type and the AZ34 mutant of *H. vulgare* L. (Table III). The enzyme activity was about 2.7-fold higher in water stressed leaves of wild type compared with that found in turgid leaves. However, similar levels of the enzyme activity were found in the turgid or stressed leaves of the AZ34 mutant. Furthermore, abscisic aldehyde oxidase activity was present only in the extracts of the wild-type leaves and no enzyme activity could be detected in the turgid or water stressed leaves of the AZ34 mutant.

**Synthesis of ABA by Cell-Free Extracts from Wilty Tomato Mutants**

We have shown earlier that cell-free extracts from wild type tomato and the wilt mutant *notabilis* readily convert xanthoxin and abscisic aldehyde to ABA while those of *sitiens* and *flacca* show little or no activity (18), suggesting that these mutants probably lack abscisic aldehyde oxidase activity. To test this, the abscisic aldehyde oxidase fraction was isolated from the bean leaf extracts by precipitating with onefold acetone and added to the cell-free extracts prepared from *sitiens* and *flacca* as described earlier (18). When 0.4 mL of these extracts was assayed with xanthoxin as the substrate, it was found that 162 ng of ABA was produced from 500 ng of xanthoxin with *sitiens* extract whereas, the *flacca* extract produced 146 ng of ABA from a total of 1000 ng of xanthoxin used as the substrate.

**Partial Purification and Some Properties of Enzymes**

Abscisic aldehyde oxidase was partially purified from fully expanded primary leaves of light-grown bean seedlings. Ten grams of the leaves were extracted with 30 mL of 50 mM KPi (pH 7.25) using a pestle and mortar kept on crushed ice. The homogenate was passed through four layers of cheese cloth and centrifuged at 12,000g for 20 min at 4°C. To the supernatant fraction thus obtained, cold acetone (1:1, v/v, −20°C) was added slowly with constant stirring. This was immediately centrifuged at 27,000g for 1 to 2 min at 4°C and the supernatant discarded. The precipitate was flushed with air to remove any residual acetone and then extracted with 50 mM KPi (pH 7.25) and concentrated five fold. This fraction containing the enzyme activity was centrifuged at 12,000g and stored frozen in small aliquots at −20°C overnight. An aliquot was thawed, centrifuged at 12,000g for 20 min at 4°C and the supernatant fraction was used for enzyme assays. Using this procedure, an enrichment of 145-fold was obtained.

Table III. Enzyme Activities in Wild-Type H. vulgare L. Steptoe and Molybdopterin Mutant AZ34

<table>
<thead>
<tr>
<th>Extract Assayed</th>
<th>Abscisic Aldehyde Formed with Xanthoxin as Substrate</th>
<th>ABA Formed with Abscisic Aldehyde as Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type unstressed</td>
<td>219</td>
<td>101</td>
</tr>
<tr>
<td>Wild-type stressed</td>
<td>591</td>
<td>131</td>
</tr>
<tr>
<td>AZ34 mutant unstressed</td>
<td>265</td>
<td>ND*</td>
</tr>
<tr>
<td>AZ34 mutant stressed</td>
<td>254</td>
<td>ND*</td>
</tr>
</tbody>
</table>

*Not detected.

Effect of pH on the activity of the partially purified enzyme was studied by varying the pH of 0.2 mM KPi buffer. As shown in Figure 1, the enzyme shows optimum activity at pH 7.25. At pH 6.0 and 8.0, the enzyme activity was 66.8% and 70.9% respectively of that found at pH 7.25.

To determine the $K_m$ and $V_{max}$ values for abscisic aldehyde oxidase, two separate experiments were conducted. For experiment I, the concentration of abscisic aldehyde was varied between 1 μM to 24 μM at a fixed concentration of NADP at 5.9 mM. The apparent $K_m$ and $V_{max}$ values, obtained from reciprocal plots, were 2.5 μM and 1 nmol ABA formed/min/mg protein respectively. For Experiment II, the concentration of abscisic aldehyde was varied between 0.25 μM to 4 μM at a fixed concentration of NADP (100 μM). The apparent $K_m$ and $V_{max}$ values obtained from reciprocal plots were 3 μM and 1.3 nmol ABA formed/min/mg protein, respectively.

To determine the $K_m$ and $V_{max}$ values for xanthoxin oxidase, coupled assays were run and the formation of ABA was followed by using 0.1 mL of fourfold acetone precipitate as the enzyme source. An additional 0.1 mL of the onefold acetone precipitate was also added to the assay system to prevent any build up of abscisic aldehyde and to ensure its complete conversion to ABA. Two separate experiments were carried out by varying the concentration of xanthoxin from 0.25 μM to 5 μM at a fixed NAD or NADP concentration of 1.5 mM. The apparent $K_m$ and $V_{max}$ values, obtained from reciprocal plots, were 3.6 μM and 0.19 nmol abscisic aldehyde formed/min/mg protein respectively for both the experiments.

After acetone precipitation, abscisic aldehyde oxidase was
stable for at least 3 weeks when stored frozen at −20°C in small aliquots. But the acetone precipitated enzyme lost more than 50% of its activity when dialyzed overnight against 20 mM KPi (pH 7.25). However, the enzyme retained 84% of its activity when 20% glycerol was introduced in the dialysis medium.

DISCUSSION

Our results reported here show that there are at least two enzymes oxidizing xanthoxin and abscisic aldehyde which are specific to their respective substrates. Xanthoxin oxidase catalyzes the conversion of xanthoxin to abscisic aldehyde whereas abscisic aldehyde oxidase converts the latter to ABA. Hence, if the synthesis of ABA occurs via the indirect pathway, both xanthoxin and abscisic aldehyde can be in the pathway. Furthermore, abscisic aldehyde seems to be the immediate precursor of ABA. The barley mutant, Az34, has been classified as a molybdenum cofactor mutant based on pleiotropic deficiencies in several enzymes (19). Molybdenum cofactor is thought to be common to all molybdoenzymes except nitrogenase (8). The involvement of a molybdoenzyme in the biosynthesis of ABA has been suggested because of the reduced capacity of the Az34 mutant with a defective molybdenum cofactor gene to accumulate ABA (22). By performing native gel electrophoresis, Walker-Simmons et al. (22) showed that the Az34 mutant lacked aldehyde oxidase activity with several substrates including abscisic aldehyde whereas the enzyme activity was present in the wild type H. vulgare. We have extended their studies and found that the wild-type Steptoe and the Az34 mutant leaf extracts both contained xanthoxin oxidase activity whereas abscisic aldehyde oxidase was present only in the wild type.

Our results are consistent with the accumulating evidence that ABA is synthesized from xanthophylls. Although xanthoxin apparently is a naturally occurring compound and is converted to ABA when fed to several plants (2, 21), it occurs at very low concentration and does not appear to be affected by water stress (2), whereas the rate of ABA biosynthesis is increased by more than 100-fold (17). Results obtained in the present study suggest that the enzymes are present in bean leaves with sufficient activity and affinity for xanthoxin and abscisic aldehyde to account for the necessary rate of ABA biosynthesis in stressed leaves provided that the supply of xanthoxin can be maintained. One possible explanation for the low levels of xanthoxin in stressed leaves may be its rapid turnover. We found that in bean leaves, exogenously fed xanthoxin is so rapidly converted to ABA that our attempts to load the leaves with xanthoxin were unsuccessful. Recently Duckham et al. (3) fed RS-[3H]abscisic aldehyde to ABA-deficient mutants of potato (droopy), pea (wilty) and Arabidopsis thaliana (ABA) and the wild-type controls and found that both the Willy and ABA mutants readily oxidized the monodeuterated abscisic aldehyde to ABA. By contrast, the droopy mutants poorly incorporated the labeled precursor into ABA and reduced the isomerized RS-[3H]abscisic aldehyde to a mixture of 2-cis and 2-trans ABA alcohols (3). The tomato mutants flacca and sitiens have been shown to accumulate substantial amounts of 2-trans-ABA alcohol (11). Hence, it is possible that instead of xanthoxin, ABA alcohol may accumulate in the stressed leaves.

Our earlier results suggest that the oxidation of xanthoxin or abscisic aldehyde does not seem to limit the rate of ABA biosynthesis in turgid leaves (17, 18). The rate limiting step may be the enzyme which cleaves xanthophylls and whose synthesis or activity is greatly increased on water stress. The resulting xanthoxin produced by such an enzyme could then be rapidly converted to ABA via abscisic aldehyde.

ACKNOWLEDGMENTS

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