The *in Vivo* and *in Vitro* Inhibition of Catalase from Leaves of *Nicotiana sylvestris* by 3-Amino-1,2,4-Triazole

Evelyn A. Havir
The Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504

**ABSTRACT**

Seedlings of tobacco (*Nicotiana sylvestris*) were treated *in vivo* with 0.03 to 20 millimolar 3-amino-1,2,4-triazole (aminotriazole). There was a rapid loss of catalase (EC 1.11.1.6) activity over the first 5 hours followed by a slower decrease for the next 4 hours to a level that was 15 to 20% of the initial activity, with little or no change for periods up to 3 days. Fifty percent loss of catalase activity occurred at 0.10 to 0.15 millimolar inhibitor (18-hour incubation). The isozymes of tobacco catalase differed in sensitivity to the inhibitor. Enhanced-peroxidatic catalase (EP-CAT) (Havir EA, McHale NA, [1989] Plant Physiol 91: 812–815) decreased 35% under conditions in which the major isozyme decreased 85%. The resistance to aminotriazole inhibition demonstrated *in vivo* by EP-CAT was also observed *in vitro*. The times for 50% inhibition at 0.67, 3.33, 5.0, 10.0, and 15 millimolar aminotriazole were 15, 5, 2.5, 2.5, and 1.5 minutes, respectively, for the major isozyme of catalase and 60, 18.5, 5.1, 4, and 3.0 minutes, respectively, for EP-CAT. Increasing H$_2$O$_2$ concentration did not change the sensitivity of EP-CAT to aminotriazole. The major form of catalase contained 4.0 ± 0.4 moles of heme per mole enzyme and EP-CAT 3.4 ± 0.3. Thus, the resistance of EP-CAT to aminotriazole is probably not due to lowered affinity for H$_2$O$_2$ or alteration in heme content but to structural changes that impair inhibitor binding.

Catalase (EC 1.11.1.6) activity is inhibited by aminotriazole$^1$ both *in vivo* and *in vitro* (2, 15, 16). The uptake, metabolism, and mode of action of the compound in plants have been studied (4–6, 18, 24), but the *in vivo* inactivation of the enzyme has seldom been investigated and there are no *in vitro* studies with a catalase of plant origin. Catalase activity was inhibited 50% in a suspension culture of pear cells after 4 h exposure to 1 mM aminotriazole (6), and was inhibited approximately 85% in rye seedlings soaked and grown in the presence of 0.25 mM inhibitor (5). Also, clones of tobacco have been selected that grow in the presence of 0.19 mM aminotriazole (25), but neither the cells nor the regenerated plants were analyzed for enzymic activities. All of these investigations were done under the assumption that there was only one type of catalase in plants.

The *in vitro* inhibition of catalase by aminotriazole has been reported by Chandlee et al. (1) for the three partially purified maize catalases that are coded for by separate genes (23). One form of catalase, CAT-3, was less sensitive to aminotriazole than the others. We have shown previously that the CAT-3 of maize exhibited a higher ratio of peroxidatic to catalatic activity than CAT-2 (12). Another possible example of aminotriazole-resistant catalase was provided by Kendall et al. (14), who found that the residual catalase activity detected on a gel after electrophoresis of an extract from a catalase-deficient mutant of barley was insensitive to aminotriazole. At the time, it was assumed that there was only one species of catalase in barley. Therefore, I considered the possibility that the resistant activity was due to the form of catalase with enhanced peroxidatic activity that we have found in barley (12).

Binding of H$_2$O$_2$ to the heme of catalase is essential for aminotriazole inhibition, although the inhibitor itself binds to the protein moiety (at histidine 74 for liver catalase) of the enzyme (15, 16, 20). A possible reason for the relative insensitivity of EP-CAT to aminotriazole might be a lower heme content, i.e., some of the four subunits of catalase might lack heme. The first crystallized preparations from spinach reportedly contained 2 mol heme/mol enzyme (8), but a later preparation had a value of 4 (9). Sunflower cotyledon catalase also contained 4 mol heme/mol (3). Although the difference in stoichiometry of binding of aminotriazole between human erythrocyte catalase and bovine liver catalase can be explained by differences in heme content, their time-dependent losses of activity appeared similar (2).

EP-CAT in tobacco has been purified and characterized (10–13). I have now investigated the inhibition of tobacco catalases by aminotriazole *in vivo* and *in vitro* to determine if EP-CAT from tobacco is insensitive to aminotriazole and whether this might occur *in vivo* as well as *in vitro*. Cytochemical localization of catalase relies on the inhibition of catalase by aminotriazole to distinguish it from peroxidase activity (7, 21). It is apparent from the results presented here that EP-CAT is resistant to aminotriazole *in vivo*, and that failure to appreciate this distinction might lead to misleading conclusions.

**MATERIALS AND METHODS**

**Enzyme Assay and Definition of Unit of Catalase Activity**

Catalase activity was determined as described previously (10). One unit of catalatic activity is defined as the amount of enzyme catalyzing the decomposition of 1 μmol H$_2$O$_2$ min$^{-1}$.  

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$^1$Abbreviations: aminotriazole, 3-amino-1,2,4-triazole; CAT-1, CAT-2, CAT-3, isozymes of tobacco leaf catalases, named in order of their elution from a chromatofocusing column; EP-CAT, enhanced-peroxidatic catalase; t$_{1/2}$, time required to reach 50% of initial activity; mU, milliunit.
Plant Material, Growth of Seedlings, Preparation of Extracts, and Enzyme Purification

Seeds of tobacco (Nicotiana sylvestris) were germinated and grown in a growth chamber as described previously (10). Extracts were prepared under standard conditions, assayed, and chromatofocused (pH 8–5) as described (10).

Inhibition of Catalase by Aminotriazole in Vivo and in Vitro

Five milliliters of aminotriazole solutions, concentrations as indicated in the figures, were added to the agar plates on which seedlings (18–21 d) were growing under a constant environment (10). The addition of 5 mL H₂O instead of inhibitor served as control. Leaves of seedlings were harvested at time intervals specified in each experiment, extracted as described previously, and assayed.

The in vitro inhibition by aminotriazole was determined with partially purified preparations of CAT-1 and CAT-3, i.e., specific activities of >10,000 and 2,000, respectively (13). Reaction mixtures consisted of 7.5 μmol KPO₄, pH 7.0, 0.15 mg glucose, 1 mM glucose oxidase, 100 to 150 units CAT-1 or CAT-3, and aminotriazole added at zero time in a total volume of 0.30 mL. The first sample, 0.025 mL, was withdrawn at 20 s and added to the standard assay mixture for immediate assay of catalatic activity. Other samples were withdrawn at time intervals indicated in the figures. The t₁/₂ value was determined graphically.

Heme Determination

Determinations were performed using the procedure of Eising and Gerhardt (3). Enzyme was purified through the chromatofocusing stages as outlined previously (13). Fractions comprising CAT-1 and CAT-3 were each combined, concentrated, and assayed for catalatic activity. A total of four separate preparations of CAT-1 and CAT-3 were tested. Each sample was assayed at several concentrations and the results averaged. With CAT-1, the reaction of enzyme with reagent showed proportionality in the range of 15 to 90 units catalatic activity. For CAT-3, 2 to 10 units catalatic activity per assay were used.

Materials

Glucose oxidase, aminotriazole, and 3,3′,5,5′-tetramethyl benzidine were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

In Vivo Inhibition of Catalase by Aminotriazole

The time course for the inhibition of catalase activity in seedlings grown in 1% CO₂ by 5 and 20 mM aminotriazole is shown in Figure 1. The inhibition is characterized by a sharp drop in activity over the first 5 h followed by a slower decrease for 4 h to a level 15 to 20% of initial activity. Activity persists essentially unchanged at this level for periods up to 3 d. A similar time course for the loss of activity in seedlings grown in air was also determined (data not shown). The treated seedlings cannot be visually distinguished from the controls.

A 16 to 18 h time period was chosen for the investigation of the effect of aminotriazole concentration on total catalatic activity because activity is unchanged during this period. The results are shown in Figure 2. In A the seedlings were grown in air and in B the seedlings were transferred to 1% CO₂ 5 d before the aminotriazole was added to increase the amount of CAT-3 in the extracts (10, 11). The concentration of aminotriazole required for 50% loss of initial activity was 100 to 150 μM, and residual activity for seedlings in air and CO₂ was 13 and 22%, respectively.

Distribution of Catalase Activity among Its Several Forms in Seedlings Treated with Aminotriazole

In Figure 3, the separation by chromatofocusing of catalases in extracts of seedlings treated with either H₂O (A) or 5 mM aminotriazole for 16 h is shown. The fractions corresponding to CAT-1, -2, and -3 (10) have been indicated. The reduction in CAT-1 plus CAT-2 and CAT-3 activities in this experiment were approximately 85 and 25%, respectively. The results from four such experiments are shown in Table 1. In all cases, CAT-1 and CAT-2 were preferentially inhibited in vivo compared with CAT-3, with an average reduction of 85% for the former and 35% for the latter. The residual activity shown in Figures 1 and 2 is largely caused by EP-CAT, and the higher value for seedlings grown in 1% CO₂ is due to the increase of this isoform that occurs on transfer of seedlings from air to 1% CO₂ (11).

In Vitro Inhibition of Catalase by Aminotriazole

The time course of inhibition of partially purified CAT-1 and CAT-3 by 5 mM aminotriazole is shown in Figure 4. Similar results have been obtained for the inhibition of liver
INHIBITION OF CATALASE FROM TOBACCO LEAVES BY AMINOTRIAZOLE

Figure 2. Effect of aminotriazole on total catalytic activity of tobacco seedlings grown in air (A) and 1% CO₂ (B). Seedlings were grown under standard conditions in air or transferred to 1% CO₂ 5 d prior to the addition of 5 mL aminotriazole (concentrations indicated). Leaves were harvested 16 to 18 h later, extracted, and extracts assayed as described in “Materials and Methods.”

Table I. Loss of CAT-1, CAT-2, and CAT-3 in Leaves of Tobacco Seedlings Treated with Aminotriazole

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>CAT-1 % of control</th>
<th>CAT-2 % of control</th>
<th>CAT-3 % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>Air</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
</tbody>
</table>

and bacterial catalases (15–17). The times required for 50% inhibition of the CAT-1 and CAT-3 for 0.67 to 15 mM aminotriazole are summarized in Table II. At every concentration, 1/2 was lower for CAT-1 than CAT-3, but the differences lessened as the concentrations increased.

The effect of increasing H₂O₂ concentration on inhibition is shown in Figure 5. Both CAT-1 (A) and CAT-3 (B) show a dependence on H₂O₂ for inhibition as demonstrated by the lack of significant inhibition when glucose oxidase is omitted from the reaction mixture. Increasing glucose oxidase fourfold did not alter the rate of inhibition of either isozyme. Thus, H₂O₂ is not the limiting factor in inhibition by aminotriazole and the differences in the rate of inhibition of CAT-1 and CAT-3 cannot be explained by the inability of CAT-3 to bind H₂O₂ as efficiently as CAT-1.

Heme Content of CAT-1 and CAT-3

The results of heme determinations on four preparations of CAT-1 and CAT-3 are shown in Table III. It is apparent that both isozymes contain approximately 4 mol heme/mol enzyme and that lower heme content does not explain the lower sensitivity to aminotriazole.

Table II. Loss of CAT-1, CAT-2, and CAT-3 in Leaves of Tobacco Seedlings Treated with Aminotriazole

<table>
<thead>
<tr>
<th>Atmosphere</th>
<th>CAT-1 % of control</th>
<th>CAT-2 % of control</th>
<th>CAT-3 % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>Air</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.67</td>
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<td>0.39</td>
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<tr>
<td>CO₂</td>
<td>0.67</td>
<td>0.40</td>
<td>0.39</td>
</tr>
</tbody>
</table>

Figure 3. Chromatofocusing of extracts of seedlings grown under standard conditions (A) and treated with 20 mM aminotriazole for 16 h before harvest (B). Conditions of growth, extraction of leaves, and chromatofocusing from pH 8 to 5 were followed as described in “Materials and Methods.”

Figure 4. In vitro inhibition of CAT-1 (○) and CAT-3 (●) by 5 mM aminotriazole. Conditions of assay are described in “Materials and Methods.”
DISCUSSION

The results show that tobacco leaf catalases are inhibited by aminotriazole in vivo, and that the isozyme of catalase with enhanced peroxidatic activity is inhibited to a lesser degree than the major catalase species. Studies with tobacco catalase isozymes in vitro confirmed the in vivo results and showed that the decreased sensitivity of EP-CAT is not due to impaired H$_2$O$_2$ binding or to alteration in heme content. The catalase-peroxidases from microorganisms, which resemble EP-CAT from higher plants in some respects, are also less inhibited by aminotriazole than other catalases (17).

The decreased sensitivity to aminotriazole shown by the enhanced-peroxidatic catalases can be due to several factors. One possibility is that binding of the inhibitor is impaired because of alterations in protein structure. Aminotriazole binds to histidine-74 of liver catalase (20), but no comparable studies to determine the site of binding have been done with a catalase of plant origin. The complete amino acid sequences for several plant catalases have been published, and it has been estimated that there is a 38% homology for the enzyme from sweet potato and rat liver enzyme (22). However, there is much stronger conservation in certain areas important in catalytic activity. Redinbaugh et al. (19) have also compared the amino acid sequences for the three maize catalases, the sweet potato, and rat liver enzymes. Again, the conclusion is that certain areas are highly conserved. Binding studies with labeled aminotriazole and the plant catalases should provide interesting information about the significant differences between the isozymes.

It can be seen from Figure 2 that even a 50-fold increase in aminotriazole over that required for 50% inhibition of initial activity does not result in complete inhibition of EP-CAT (Table I). In contrast, whereas significant differences in the rates of inactivation in vitro of CAT-1 and CAT-3 are noted, these differences become much less apparent at the higher concentration of aminotriazole (Table II). Thus, there may be reasons aside from changes in protein structure for the insensitivity of CAT-3 to aminotriazole in vivo. One possibility is that EP-CAT, in vivo, is not accessible to aminotriazole. This would indicate a nonperoxisomal location for the enzyme because the major form of catalase is located in this organelle.

Table II. Time for 50% in Vitro Inhibition of CAT-1 and CAT-3 at Varying Aminotriazole Concentrations

<table>
<thead>
<tr>
<th>Aminotriazole (mm)</th>
<th>$t_{1/2}$ CAT-1 (min)</th>
<th>$t_{1/2}$ CAT-3 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.67</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>3.33</td>
<td>5</td>
<td>18.5</td>
</tr>
<tr>
<td>5.0</td>
<td>2.6</td>
<td>5.1</td>
</tr>
<tr>
<td>10.0</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>15.0</td>
<td>1.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Another possibility is that EP-CAT is localized at a site in which there is a limiting amount of H$_2$O$_2$. This explanation has been evoked by Margoliash et al. (16) to explain the observation that in mammals the in vivo inhibition of catalase by aminotriazole occurs in the liver and kidney but not in blood. Previous results for the peroxidatic reaction (12) and those presented in Figure 5 indicate that the binding of H$_2$O$_2$ by both EP-CAT and the major catalase species is similar.

In conclusion, this study confirms my earlier work indicating that the isozymes of catalase that exhibit enhanced-peroxidatic activity have similar properties, whether the source of the enzyme is tobacco, maize, or barley. Further, the assumption that all activity that is measured in the presence of aminotriazole is due to a peroxidase, not catalase, must be challenged.

Table III. Heme Content of CAT-1 and CAT-3

<table>
<thead>
<tr>
<th>Preparation</th>
<th>CAT-1 (mol heme/mol catalese)</th>
<th>CAT-3 (mol heme/mol catalese)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.1</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
<td>3.5</td>
</tr>
<tr>
<td>3</td>
<td>3.4</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>3.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Average</td>
<td>4.0 ± 0.4</td>
<td>3.4 ± 0.3</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

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LITERATURE CITED

18. Racusen D (1957) The metabolism and translocation of 3-amino-1,2,4-triazole in plants. Arch Biochem Biophys 74: 106-113