Assay for *Helminthosporium maydis* Toxin-binding Activity in Plants

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ABSTRACT

A relatively rapid and sensitive assay is described for assessing the binding of *Helminthosporium maydis* Race T's C-toxins I and II to plant components. The technique is a modification of the one of Haddad and Birge (J. Biol. Chem. 250: 299–303, 1975), and utilizes dextran-coated charcoal as an adsorbent for the unreacted toxin and employs a Millipore filter to isolate the protein-toxin complex.

Extracts from susceptible corn line W64A Tms possess a protein primarily localized in the cytosol which is relatively heat-insensitive, flicin- and papain-sensitive, and binds toxins I and II at half-saturation in the order of 0.1 mM. The toxin-binding activities of the extracts of various corn lines and other species are not correlated to resistance or susceptibility to *H. maydis* Race T, nor to sensitivity to the toxins. These findings are discussed relative to the function of the binding protein and cellular sensitivity to the toxins.

*Helminthosporium maydis* Race T is the causal agent of southern corn leaf blight. Numerous reports have been published showing that *H. maydis* produces a toxin or toxins in culture (6, 8, 9, 15, 23). Furthermore, these toxins are host-specific, i.e. they affect the same hosts as does the fungus which produces them. Karr et al. (9, 10) purified five of these toxins extensively and showed that they were all basically terpenoids and derivates thereof. All five toxins were isolated from leaves of fungus-infected corn of variety W64A Tms and the compounds could not be recovered from inoculated resistant variety MO-17 N (9), suggesting that these compounds play a vital role in producing disease symptoms.

Toxin preparations of *H. maydis* Race T have been shown to stimulate the oxidation of malate + pyruvate, inhibit phosphofructokinase, and induce swelling of mitochondria from susceptible but not resistant plants (12). Toxin preparations have also been shown to cause ion leakage, reduce ATP content, inhibit root growth, inhibit photosynthesis, and have a direct effect on stomatal function (1, 2). The inhibition of membrane ATPase activity has also been noted (20). Many of these experiments have been conducted with relatively crude toxin preparations, therefore making the results difficult to evaluate.

Recently, Watrud et al. (22) showed that malate dehydrogenase was inhibited in mitochondria from Tms corn, but not in mitochondria from N cytoplasm corn (line W64A). However, when the outer mitochondrial membrane was ruptured or removed, the mitochondria in the corn line bearing N cytoplasm became sensitive. The workers suggested that a permeability barrier existed in the N cytoplasm. Toxin-binding studies showed that the corn mitochondria of both N and Tms cytoplasm bound toxins II and I, but that the greatest binding activity was associated with the inner mitochondrial membrane of Tms corn. These results are first to show a direct toxin interaction with a component of the plant cell. The binding assays conducted by these investigators relied upon the bioassay utilizing toxin-induced effects on sensitive mitochondria which involves laborious procedures. The purpose of this report is to describe a rapid toxin-binding assay which utilizes a mixture of C-toxins I and II and dextran-coated charcoal in order to facilitate toxin-binding assays in plant extracts. We also demonstrate the nature, extent, and distribution of toxin-binding activity in plants.

MATERIALS AND METHODS

Toxin Preparation. The toxin preparations used throughout these experiments were obtained by the procedures outlined by Karr et al. (9). The toxins were taken through the paper chromatography step followed by extraction of the toxins into acetone. TLC of the acetone-soluble toxins revealed primarily toxins I and II (9). Toxins I and II were prepared radiolabeled with 14C by administering cultures [U-14C]acetate according to the methods of Karr et al. (9). The specific activities of the toxin I and II mixture varied from 1.09 to 1.42 × 106 dpm/μmol (mol weight to be taken as 388).

Leaf Bioassay. The test for the biological activity of toxin samples was assessed by a modification of the procedure described by Bhullar et al. (3). Second and third leaves from plants in the five-leaf stage were cut crosswise into thirds and each piece placed into a 10-ml beaker containing 0.6 ml of H2O. Between 3 and 10 μl of the toxin preparation dissolved in acetone was added to the water in the bottom of the beaker. The small beaker containing the leaf pieces was then placed in a 1-liter beaker with a reservoir of H2O, and the container sealed with Saran Wrap. It was then placed in a growth chamber, 500 ft·c at 28 C. Characteristic water-soaking and gray-brown discoloration of toxin-treated leaves generally appeared at 18 hr after treatment and the symptoms were fully developed by 24 hr.

Thin Layer Chromatography. Precoated Silica Gel 60 plates (0.25 mm) prepared by E. Merck, Darmstadt, Germany were used for TLC in a solvent system consisting of n-propanol-ethyl acetate-H2O, 7:2:1, v/v. The solvents were distilled prior to use. The plates were sprayed with a reagent consisting of 5% H2SO4 in ethanol and heated at 110 C for several min to reveal the position of the toxins. On these plates the Rf values of toxins I and II were 0.84 and 0.72, respectively.

Preparation of Leaf Extracts. Leaf tissue (1 g wet weight) was taken from plants that were between 6 weeks and 3 months old. The tissue was cut into several pieces and ground in a mortar with a liberal amount of liquid N2. The frozen powder was
transferred to a beaker and 5 ml of 50 mM tris·HCl buffer (pH 7.5) was added. The powder was stirred gently to wet it completely and the solution was filtered through cheesecloth. The filtrate was centrifuged at 39,000 g for 20 min and the supernatant liquid was carefully recovered by pipetting with a Pasteur pipette. The recovered supernatant liquid was used directly for toxin-binding assays.

Toxin-binding Assay. The assay for toxin-binding was adapted from the procedure of Haddad and Birge (5). A standard assay contained 0.75 ml (0.75 μmol) of the original plant extract, or dilutions thereof, in a plastic tube. An aliquot of the stock solution of the 14C-toxin preparation containing 0.1 μmol (10,000 dpm) in 10 μl of acetone was pipetted directly into the plant extract. Acetone must be used as the solvent since this toxin is only slightly soluble in H2O. The contents of the tube were incubated for 30 min at 22°C. The reaction was terminated by the addition of 0.25 ml of dextran 20-coated charcoal (7) and stirred thoroughly with a Vortex mixer. The mixture was incubated for 10 min to allow for the complete adsorption of the unbound toxins by the charcoal. The charcoal was removed by filtering the suspension through a Millipore filter (HAWP 0 2500) which was supported by a stainless steel disc. The filter was washed with 0.5 ml of 50 mM tris·HCl (pH 7.5) and the filtrate, which contained the plant product-toxin complex, was collected. Radioactivity in the filtrate was determined in Aquasol (New England Nuclear) and cpm determinations were corrected to dpm by the linear quench curve method. To get reproducible results on any given preparation for binding activity, the binding assay must be done as soon after the preparation of the plant extract as possible. All data points shown are the average of at least two determinations.

Soluble plant protein was determined after exhaustive dialysis against the tris·HCl buffer, by the method of Lowry et al. (11) using BSA as a standard.

Materials. Inbred corn lines Mo17 and 33-16 were obtained from M. S. Zuber, University of Missouri; lines W64A from V. E. Gracen, Cornell University; and lines Mo/10ae from P. Petersen, Iowa State University.

All 14C-labeled compounds were acquired from Amersham-Searle possessing activities between 53 and 60.7 mCi/mmol. Enzymes, sterols, dextran 20, and activated charcoal were purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

Validity of the Assay for Toxin-binding. Radioactivity attributed to nonspecific binding was determined from samples containing BSA (about 155 dpm) at the same protein concentration—1 mg/ml and in the same buffer as contained in the leaf extract preparations. The assay was considered highly sensitive to specific toxin-binding activity since the control value was only 5 to 10% of the values for toxin binding by corn leaf extracts. It was clear that little toxin was bound nonspecifically to BSA since 99% of the BSA passed through the filter, A at 260, and the control value also includes any 14C not absorbed by the charcoal. The variation between duplicate assays on any given preparation was between 5 and 10%; however, the variation between different extracts from the same plant averaged ±20%.

A leaf extract preparation (1.5 ml, 1.5 mg protein) from line W64A Tms corn was incubated with 0.2 μmol of the 14C-toxins. After passage through the charcoal-dextran filter, the filtrate was extracted twice with 3 ml of 1-butanol and the combined butanol fractions obtained after centrifugation at 10,000g were evaporated and dissolved in acetone. The total material obtained was tested in the leaf bioassay and characteristic toxin-induced symptoms appeared on the leaf. In another identical experiment, the butanol-extracted material (4050 dpm) was chromatographed by TLC and 60% of the recoverable radioactivity appeared at the same Rf values as authentic toxins I and II with approximately equal amounts of labeling in toxins I and II. TLC of the standard 14C-toxin preparation revealed a 61% recovery of radioactivity in toxins I and II with nearly equal amounts of labeling in both compounds. The radioactivity not directly recoverable as the toxins is associated with either "tailing" or breakdown products of toxin II on the chromatogram, as a band that moves slightly faster than toxin I, and also as material lost during chromatography.

Toxin-binding Activity. A leaf extract of corn line W64A Tms containing 1 mg/ml of protein was diluted with the tris·HCl buffer (pH 7.5) to yield the protein concentrations shown in Figure 1. The amount of toxin bound was a linear function of protein concentration up to 800 μg protein/ml (Fig. 1). The pH optimum for toxin-binding ranged from pH 7.5 to 7.8. When the protein concentration was held constant and the concentration of the toxin was varied, the amount of toxin bound followed a sigmoidal relationship (Fig. 2). The approximate binding constant for the toxin in this relationship obtained by estimating 50% of maximal toxin-binding is in the order of 0.1 mM.

The specificity of the plant extract for the binding of various sterols was tested using 0.1 μmol of each 14C-sterol in the assay system. Relative to the toxin, the binding activity of the plant extract for other sterols tested including progesterone, and cholesterol was only a small fraction of that normally observed for the toxins.

Nature of Binding Activity. A number of experiments were conducted in order to ascertain the nature of the toxin-binding activity in plant extracts. For instance, the binding activity is resistant to heating at 80 to 85°C for 15 min, one-half of the activity in the crude extract is lost after the incubation at 37°C for 4 hr. However, this loss in activity is not the result of the instability of the binding activity to prolonged exposure to heat, because the sample incubated with 1 mg of pronase under the same conditions retained all of its binding activity. The fact that pronase protects the binding activity during prolonged heat treatment indicates that it degraded some enzymic activity which would have otherwise neutralized the toxin-binding activity. The toxin-binding activity itself is resistant to pronase digestion.

Papain and ficin, broad specificity plant proteases, have lesser abilities to protect the binding activity from degradation. These two enzymes are activated by cysteine (13, 21) and were used at a level of 1 mg/ml. A comparison of the papain-treated sample with its control indicates that the degrading activity is also sensitive to papain; however, it was not possible to ascertain whether a 20% loss of binding activity in the papain sample was caused by the action of papain or by the degradative activity present in the crude plant extract. The same reasoning applies to the 50% loss in activity obtained with ficin.

To evaluate further the sensitivity of the binding activity to proteolytic enzymes, the binding activity was separated from its degrading activity by heating the extract at 80 to 85°C for 15 min. The binding activity was less sensitive to heating at 37°C for 4 hr than previously because of the removal of the degradative...
activity. Subsequently, the toxin-binding activity became sensitive to ficin at this point since 80% of its activity was destroyed in 4 hr with 1 mg/ml ficin. The data suggest that some critical component of the toxin-binding activity contains ficin-susceptible peptide bonds. Because the binding activity is heat-insensitive, a workable first step in purification of the toxin-binding activity is heat treatment at 80 to 85 °C for 15 min. At least a 5-fold purification in activity does result after such treatment. Furthermore, another 5-fold increase in specific binding activity results after (NH₄)₂SO₄ precipitation (30–50% saturation); however, only 7% of the total activity remains.

**Specificity of Toxin-binding.** A survey of corn lines was made in order to determine if a relationship exists between tissue susceptibility to the *H. maydis* toxins and toxin-binding activity. When compared on a specific activity basis, all corn lines tested showed toxin-binding activity, however there were slightly elevated activities in the Tms versions of W64A, 33-16 and M017 (Table I). In the bioassay test, the most susceptible corn line is W64A Tms, but the N version of W64A is also susceptible to the toxin. The most toxin-resistant line is M010ae N; it bound more toxin/mg protein than any of the other extracts tested. From these results, we conclude that there is no correlation between toxin-binding per se and tissue resistance or susceptibility to the *H. maydis* toxins. In this regard, this system is quite unlike that observed for toxin susceptibility in sugarcane to helminthosporiose (16, 17). This does not preclude the importance of toxin-binding to some additional structurally controlled event that is inextricably associated with tissue susceptibility.

**Site of Action of the Toxins.** The crude membrane fraction of W64A Tms (39,000g pellet) has toxin-binding activity that is extractable with m-trichloroacetate-tris-HCl, 50 mM buffer (pH 7.5). The crude soluble membrane protein has relatively the same specific activity for toxin-binding as the protein from the cytosol of the plant tissue, but it has an approximately 100-fold less binding activity/g tissue. This could be due to the presence of a toxin-binding protein in a cellular membrane or some contamination with the cytoplasm. Since increased susceptibility to the toxin in most cases is cytologically inherited, attention is directed to the mitochondria and chloroplasts. Thus far, no direct effect of the toxin on chloroplast function has been demonstrated. However, differential permeability between N and Tms mitochondria has been shown (22). Leakage studies by several investigators (2, 4) would also indicate that another site of toxin action may be at the plasma membrane.

The availability of the ¹⁴C-toxin-binding assay should enable investigators ultimately to purify, characterize, and localize the toxin-binding site (5) and determine its role in the toxin reaction. This approach would supplement the work which has been conducted on the effects of the toxin on specific enzymes or functions associated with intact organelles or tissues.

As far as we know, there have been no other comparable binding studies in plant or animal systems which involve a tetrapeptide-like toxin. However, the extensive work which has been done on the mammalian steroid hormones and their subunit protein receptors may provide an analogy (14). There is some suggestion from the data that the toxin-binding protein in corn also is a subunit protein (Fig. 2). The sigmoidal nature of the saturation curve suggests a cooperative subunit interaction and multiple toxin-binding sites (Fig. 2). Further, the drastic loss in activity after (NH₄)₂SO₄ precipitation may have resulted from salt-induced subunit dissociation. The association of the toxins to the protein is relatively low when compared to the K₅ values of the steroid hormones. Any number of factors could contribute to this phenomenon including the confirmation of the binding protein and the influence of contaminating proteins.

Although toxin-binding occurred in extracts of plant tissues other than corn (Table I), this is not to negate the fact that the toxin-protein interaction in susceptible corn is a vital factor in toxin-induced symptom production. It is conceivable that the toxin-binding protein mediates some normal function in the healthy plant. Precedent for this example is to be found in the plasma membrane-localized binding protein of sugarcane, whose apparent role is in α-galactoside transport (18, 19).

**LITERATURE CITED**

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**Table I.** The binding of *H. maydis* toxins I and II to the extracts of various corn lines and other plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Binding Activity (dpm)</th>
<th>0.1 M Tris-HCl 0.1 M NaCl 0.001 M phenol reagent</th>
<th>0.1 M Tris-HCl 0.1 M NaCl 0.001 M phenol reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>W64A – N</td>
<td>1596 ± 260</td>
<td></td>
<td></td>
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<tr>
<td>W64A – Tms</td>
<td>2025 ± 715</td>
<td></td>
<td></td>
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<tr>
<td>33-16 – N</td>
<td>1493 ± 103</td>
<td></td>
<td></td>
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<tr>
<td>33-16 – Tms</td>
<td>1640 ± 459</td>
<td></td>
<td></td>
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<tr>
<td>No17 – N</td>
<td>1796 ± 137</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No17 – Tms</td>
<td>1991 ± 518</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M010ae – N</td>
<td>2442 ± 337</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M010ae – Tms</td>
<td>1995 ± 407</td>
<td></td>
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<tr>
<td>Sugarcane 51 BG 97</td>
<td>1826 ± 542</td>
<td></td>
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<tr>
<td>Potato russet-burbank</td>
<td>1062 ± 154</td>
<td></td>
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<tr>
<td>Tobacco N. tabacum</td>
<td>593 ± 130</td>
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</tbody>
</table>
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