Corn Seed Proteins Inhibitory to *Aspergillus flavus* and Aflatoxin Biosynthesis

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Accepted for publication 12 March 1997.

ABSTRACT

Huang, Z., White, D. G., and Payne, G. A. 1997. Corn seed proteins inhibitory to *Aspergillus flavus* and aflatoxin biosynthesis. *Phytopathology* 87:622-627.

This study reports the presence of two fractions from corn seeds inhibitory to aflatoxin formation. Using a sensitive laboratory assay that can measure both inhibition of fungal growth and inhibition of aflatoxin biosynthesis, we examined aqueous extracts from seeds of Tex6, a corn inbred shown to be highly resistant to aflatoxin accumulation in field and laboratory evaluations. In these extracts, we identified two biologically active fractions. One inhibited growth of *Aspergillus flavus* and, thus,

aflatoxin accumulation, and the other inhibited aflatoxin formation with little effect on fungal growth. The compounds responsible for these activities appear to be proteaceous, as they are water soluble, heat labile, and sensitive to proteinase K treatment. The compounds were partially purified by ultrafiltration and chromatography. The estimated molecular mass of the growth inhibitor is approximately 28 kDa, and that of the aflatoxin biosynthesis inhibitor appears to be greater than 100 kDa. Partially purified preparations of the growth inhibitor and aflatoxin biosynthesis inhibitor cause 50% inhibition at 26 and 75 µg of protein/ml, respectively. The presence of these compounds in Tex6 may explain its resistance to aflatoxin accumulation.

Preharvest contamination of corn (*Zea mays* L.) with aflatoxin, a carcinogenic fungal secondary metabolite, is a recurrent problem in the southeastern United States (3,29,34,40,41). In recent years, aflatoxin contamination also has become a problem in the Midwest region of the United States (40). Postharvest elimination of aflatoxin from corn kernels is not economically feasible and, thus, research efforts are focused on control strategies that prevent preharvest contamination. The most desirable control strategy is the use of resistant corn hybrids (29). Although resistance to aflatoxin accumulation is a heritable trait (7,8,9,10,19,22,29,33), the screening for resistant genotypes has been complicated by the inherent year-to-year variation in aflatoxin contamination in field studies. Further, field evaluations for resistance to aflatoxin accumulation can be conducted only once a year, or twice a year with the use of a winter nursery. For these reasons, the ability to identify resistance using laboratory assays or to associate resistance with specific compounds in resistant corn kernels could greatly facilitate a breeding program.

Several attempts have been made to develop laboratory assays for the evaluation of resistance to aflatoxin accumulation in corn (2,23,39). In these assays, which were mainly based on the inoculation of individual kernels with *Aspergillus flavus*, it was often possible to observe differences among genotypes, but these differences were not always consistent over years or sampling dates. None of these assays are currently being used. In a 1983 review, King and Wallin (23) stated that one factor that would greatly facilitate the screening of corn genotypes for resistance to aflatoxin accumulation is the identification of at least one resistant genotype to serve as a benchmark for the reliability of any laboratory screening technique. Recently, corn genotypes with resistance to aflatoxin accumulation have been identified in field studies (7,8,10,19,29,33). Brown et al. (5) were able to test the reliability of

their laboratory assay by testing genotypes of corn differing in resistance to aflatoxin accumulation. They developed an assay that measures colonization and aflatoxin accumulation in detached inoculated corn kernels. Using this assay, they were able to show a relationship between resistance to fungal growth in detached seeds and resistance to aflatoxin contamination in field evaluations. This assay, however, does not lend itself to the identification of inhibitory compounds present in seeds.

There have been several attempts to identify inhibitory compounds in corn kernels associated with resistance to aflatoxin biosynthesis and fungal growth, with the intent of using these compounds in the control of aflatoxin contamination (16,25-28). Norton (28) reported nonpolar compounds from corn kernels to be inhibitory to aflatoxin accumulation and fungal growth, and Duvick et al. (16) identified several small basic peptides from corn kernels with novel antimicrobial activities. Nagarajan and Bhat (25) found extracts from an Opaque-2 corn line that almost completely inhibited aflatoxin formation. However, no further results have been reported on the characterization of this active compound and its association with resistance to aflatoxin accumulation in the field. Therefore, in neither these studies nor those of others (26, 27) has a significant association been shown between enzyme activities or proteins in corn kernels and field resistance to *A. flavus*.

The overall objective of the research reported here was to use a laboratory bioassay to examine a resistant corn genotype for the presence of compounds inhibitory to growth of *A. flavus* or to aflatoxin biosynthesis. We present results to show that the assay we have developed can be used to identify compounds inhibitory to fungal growth and to aflatoxin accumulation. We also report the initial characterization of a compound inhibitory to growth of *A. flavus*. Preliminary results have been reported (20,21).

MATERIALS AND METHODS

Corn seeds and fungal strains. The corn genotypes Tex6 and B73 used in this study were grown in Champaign, IL, in 1994, or in North Carolina in 1995. The seeds were dried and then stored at

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Publication no. P-1997-0407-02R

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room temperature. The genotype Tex6 has been proven to be a resistant inbred in field (9) and laboratory studies (5). B73 is less resistant than Tex6 and ranges from susceptible (9) to moderately resistant (5) in evaluations for resistance. *A. flavus* strain 13-22 (*white*, *leu*, and *aflR*⁺), derived from strain 656-2, was used in all of these studies (17). A sister strain, 2-4, has been shown to colonize corn tissue similar to a wild-type strain (5). Wild-type strains NRRL 6539 and NRRL 6540 were obtained from the National Center for Agricultural Utilization Research in Peoria, IL.

Laboratory assay procedures. Corn kernels were ground in a coffee mill (Mr. Coffee, Inc., Bedford Heights, OH), and the resulting powder was extracted immediately or stored at -20°C . An extract of the kernels was prepared by soaking the powder (1:5 wt/vol) at 4°C for 4 to 6 h in distilled water, with four vigorous agitations during the extraction. The resulting extract was filter-sterilized through a $0.45\text{-}\mu\text{m}$ filter and used immediately in the assay or stored at -20°C until used. Three parts kernel extract were added to one part sucrose low salts (SLS) medium, a medium conducive for aflatoxin production (6,30). In all experiments, 25% SLS was used as the control medium. Two hundred-microliter aliquots of the extract in SLS medium were distributed into 96-well tissue culture plates (Becton Dickinson Company, Franklin Lakes, NJ), inoculated with conidia of *A. flavus* strain 13-22 to a final concentration of 2×10^5 conidia per milliliter, and incubated at 28°C for 84 h.

Aflatoxin B₁ concentrations in the medium were determined by an indirect competitive enzyme-linked immunosorbent assay (ELISA) described by Chu et al. (12) and Gendolff et al. (18). All reagents were purchased from Sigma Chemical Co. (St. Louis). Fungal growth was determined by weighing lyophilized fungal mats.

Characterization of the corn extracts. *Temperature sensitivity.* Aqueous kernel extracts were heated in a water bath at 40, 65, or 100°C for 10 min and centrifuged for 3 min at $16,000 \times g$. The supernatants were assayed as described above for their ability to inhibit aflatoxin accumulation. Tex6 extract, held at room temperature, served as a positive control.

Solubility. The aqueous extract from Tex6 was partitioned three times with equal volumes of ethyl acetate, and the ethyl acetate phase, water phase, and the interface were collected separately. The ethyl acetate from the ethyl acetate phase and interface was removed under reduced atmospheric pressure at room temperature, and the residues were redissolved in water up to the original extraction volume. Residual ethyl acetate in the aqueous phase was removed under reduced atmospheric pressure at room temperature for 30 min. Aqueous extracts of the three phases were assayed as described above for inhibition of aflatoxin B₁ production. To determine if the inhibitory activity could be partitioned into methanol, methanol was used to extract a lyophilized kernel extract. After extraction, the methanol was removed under reduced pressure at room temperature, and the residue was redissolved in water and assayed as described above.

Fractionation with $(\text{NH}_4)_2\text{SO}_4$. $(\text{NH}_4)_2\text{SO}_4$ solutions at 40 and 50% saturations were prepared as described (14). Five milliliters of corn extract was added to dialysis tubing, having a molecular mass cut off (MMCO) of 6 to 8 kDa. The extract was dialyzed against 500 ml of water overnight and then dialyzed overnight in 500 ml of a 40% saturated $(\text{NH}_4)_2\text{SO}_4$ solution. Precipitates resulting from treatment with the 40% saturated $(\text{NH}_4)_2\text{SO}_4$ solution were collected by centrifugation and redissolved in 50 mM Tris-HCl buffer, pH 6.5. The supernatant from the 40% saturated $(\text{NH}_4)_2\text{SO}_4$ treatment was dialyzed against 500 ml of a 50% saturated $(\text{NH}_4)_2\text{SO}_4$ solution overnight. Precipitates resulting from this treatment were collected by centrifugation as described above. The remaining supernatant was dialyzed against 1,000 ml of water for 4 h and dialyzed in 4,000 ml of 50 mM Tris-HCl buffer along with the reconstituted precipitates from the 40 and 50% saturated $(\text{NH}_4)_2\text{SO}_4$ treatments. These preparations were assayed for inhibition of aflatoxin B₁ production as described above.

Proteinase treatment. Corn extracts were incubated with proteinase K (Sigma Chemical Co.) at 12.7 units/ml in 50 mM Tris-HCl buffer plus 2 mM CaCl_2 (pH 7.5) for 2 h at 37°C , and assayed for inhibition of aflatoxin biosynthesis using the laboratory assay described above. Seed extracts incubated with inactivated (100°C for 10 min) proteinase K were used as controls. A mixture of 25% SLS medium and proteinase K was also used as a control.

Chromatographic characteristics of the inhibitory compounds. *Anion exchange chromatography.* Ten milliliters of seed extract in a buffer of 10 mM Tris-HCl, pH 8.0, was applied to a column filled with 25 ml of DEAE Sepharose CL-6B (Pharmacia Biotechnology Inc., Uppsala, Sweden) preconditioned in 10 mM Tris-HCl, pH 8.0. The column was eluted at a flow rate of 16 ml/h. A preelution wash with 75 ml of the buffer was followed by elution with a linear gradient from 0 to 1 M NaCl. Three-milliliter fractions were collected and dialyzed overnight against water, sterilized by filtration, and assayed in SLS medium for inhibition of aflatoxin B₁ biosynthesis and fungal growth.

Hydrophobic interaction chromatography (HIC). Fractions containing inhibitory activities from the above anion exchange column were combined and applied on a methyl HIC column (Bio-Rad Laboratories, Hercules, CA). Application conditions were as described in the manufacturer's instruction. The column was eluted with a linearly decreasing gradient ranging from 1.5 to 0.0 M $(\text{NH}_4)_2\text{SO}_4$. One-milliliter fractions were collected, and certain fractions were pooled based on the protein elution profile. These pools were dialyzed against 4 liters of water overnight and lyophilized. The fractions were resuspended in 1 ml of 25% SLS medium to assay for inhibition of fungal growth and aflatoxin biosynthesis.

Filtration chromatography. Centricon ultrafiltration devices (Amicon, Inc., Beverly, MA) with MMCOs of 10, 30, 50, or 100 kDa were used to separate the two inhibitory compounds and estimate their molecular masses. The conditions for the ultrafiltration were described in the manufacturer's product instruction. Two milliliters of Tex6 extract was applied to a Centricon of 100-kDa MMCO and concentrated until approximately 0.3 ml was retained in the upper part of the Centricon. This material was washed and centrifuged twice with 1 ml of extraction buffer. Filtrate that passed through the 100-kDa MMCO Centricon was applied to a 50-kDa MMCO Centricon and processed as with the 100-kDa MMCO Centricon. This process was repeated with the 30- and 10-kDa MMCO filters. The fractions retained by Centricon filters were calibrated to the starting volume of 2 ml with extraction buffer and bioassayed as described above. A gel filtration column of 1 cm in diameter and 100 cm in length, filled with Bio-Gel P-100 (Bio-Rad Laboratories) was used to further fractionate the inhibitory compounds prepared from the Centricon filters. The flow rate of the elution buffer, 50 mM Tris-HCl (pH 6.8) and 200 mM NaCl, was 15 ml/h. Fractions of 1 ml each were added to peptone mineral salt (PMS) medium, a medium that supports good growth of *A. flavus* (6) by mixing one part of four-times-concentrated PMS medium to three parts of the fraction. Inhibition of fungal growth was determined using the laboratory assay described above. After 84 h of growth, the mycelium was washed with deionized water, lyophilized, and weighed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was conducted as described (15). Samples were treated with 2% SDS at 65°C for 5 min with or without 2% β -mercaptoethanol. The gel was stained with a Bio-Rad silver stain kit (Bio-Rad Laboratories) according to the recommendations of the manufacturer. The molecular mass of the proteins was estimated by comparison with Bio-Rad standards.

Quantification of inhibitory activity. Extracts of Tex6 retained on the 50-kDa MMCO Centricon filter were serially diluted in PMS medium and assayed for inhibition of fungal growth as described above. A fraction obtained from DEAE anion exchange chromatography with activity highly inhibitory to aflatoxin production was used to quantify the inhibitory activity. This inhibi-

tory fraction was serially diluted in SLS medium and assayed as described above. Aflatoxin production was determined by ELISA, and mycelia were collected, washed with deionized water, and lyophilized for dry weight measurement. Regression analyses between protein concentration and mycelial growth or aflatoxin accumulation were performed.

Chitinase assay. Ten percent water agar containing 2% chitin was used to assay for chitinase activity in the crude seed extract and in the Centricon preparation that inhibited fungal growth. Five microliters of sample was placed on the surface of the chitin agar plate, and the plate was incubated at 28°C. Chitinase at 1 unit/ml, purchased from Sigma Chemical Co., was used as a positive control.

Statistical analyses. All treatments were replicated two to four times. Analyses of variance were conducted to test for differences among different treatments. Student's *t* tests were applied for pairwise comparison when significant differences were found.

RESULTS

Inhibitory activity in extracts of Tex6. The addition of aqueous extracts of seeds of inbred Tex6 to SLS medium reduced aflatoxin production by *A. flavus* by 98%. In contrast, the addition of aqueous extracts of B73 reduced aflatoxin production by 61% (Table 1). Mycelial growth of *A. flavus* on extracts of Tex6 was only 39% of that on extracts of B73. These data suggested to us that mature seeds of Tex6 contain a compound(s) inhibitory to growth and aflatoxin accumulation.

To determine the nature of the inhibitory activity found in Tex6, a series of experiments were designed to characterize the physical properties of the compound(s) associated with this activity. The bioassay we used was sensitive and allowed small sample volumes to be evaluated. Both of these characteristics made it possible to easily follow the inhibitory activity present in seed extracts during its purification. Because our initial interest was to identify

compounds that inhibit aflatoxin accumulation, in our initial assays, we measured only aflatoxin production and not fungal growth. The inhibitory activity in Tex6 extracts was abolished after 10 min at 100°C, but only slightly reduced after 10 min at 40°C (Fig. 1). The inhibitory activity did not follow compounds that partitioned in any organic solvent tested. It did not partition into ethyl acetate or the interface between ethyl acetate and water. Further, treatment of lyophilized seed extracts with methanol reduced the inhibitory activity. The inhibitory activity was associated with compounds precipitated by 50% saturated $(\text{NH}_4)_2\text{SO}_4$ solutions, but not in the precipitates from 40% saturated $(\text{NH}_4)_2\text{SO}_4$ solutions.

The extract was assayed for chitinase activity, but no chitinase activity was observed in Tex6 extracts after 1 week of incubation at 28°C (data not shown). A clear zone appeared where the chitinase standard was applied to the chitin agar after 12 h of incubation at 28°C. The zone of clearing expanded to cover the entire plate by the end of the assay.

Chromatography of the inhibitory extracts. Fractionation of Tex6 extracts by anion exchange chromatography revealed fractions eluting at two different ionic strengths that inhibited aflatoxin production in *A. flavus* (Fig. 2). A fraction eluting at 200 mM ion strength resulted in the greatest inhibition of aflatoxin production, while fractions eluting at 240 mM ion strength resulted in the greatest inhibition of fungal growth. These data indicated the presence of at least two inhibitory compounds in Tex6, one inhibiting aflatoxin biosynthesis and the other one inhibiting fungal growth. Fractions eluted between 175 to 240 mM ion strength were collected, pooled, and subjected to HIC. As shown in Figure 3, fractions eluted by 0.6 to 0.86 M $(\text{NH}_4)_2\text{SO}_4$ (fraction 4) inhibited fungal growth (and, therefore, aflatoxin production), while fractions eluted by 0.11 to 0.16 M $(\text{NH}_4)_2\text{SO}_4$ (fraction 8) inhibited aflatoxin production, but had little effect on fungal growth. We have designated the former fraction the *A. flavus* growth inhibitor (AFGI) and the other fraction the aflatoxin biosynthesis inhibitor (ABI).

The two activities could also be separated by ultrafiltration using Centricon filters. The AFGI was able to pass through the 100-kDa MMCO Centricon, but was retained by a 50-kDa MMCO Centricon, suggesting the putative size of the AFGI to be larger than 50 kDa, but smaller than 100 kDa. The ABI was retained by

TABLE 1. Inhibition of aflatoxin production and fungal growth by corn extracts

Seed extracts	Aflatoxin B ₁ (ng/ml)	Fungal growth (mg)
Control (25% SLS) ^y	1,196 a ^z	0.4 c
B73	462 b	1.8 a
Tex6	20 c	0.7 b

^y SLS = sucrose low salts.

^z Numbers labeled with different letters are significantly different from each other (*P* = 0.05).

Fig. 1. Temperature stability of the inhibitory activity present in seed extracts of Tex6. Extracts were incubated at the temperatures indicated for 10 min, and then assayed for inhibitory activity. Numbers above bars represent the mean aflatoxin concentration of each treatment. Error bars represent standard errors of four replicates of each treatment.

Fig. 2. DEAE anion exchange chromatography of seed extracts of Tex6. The dotted curve represents the protein elution profile as measured by absorbance at 280 nm. Bars represent aflatoxin production by *Aspergillus flavus* in respective fractions collected from the column and assayed as described in Materials and Methods. The solid line represents the mycelial dry weight of *A. flavus* after growth in the fractions. Aflatoxin and mycelial dry weight values are the means of two replicates. Error bars represent standard errors of the two replicates. Inhibitory activities to aflatoxin production and fungal growth eluted at 175 to 240 mM ion strength.

a 100-kDa MMCO Centricon, suggesting a molecular mass greater than 100 kDa. There was no aflatoxin inhibition by fractions of corn inbred B73 retained by the 100-kDa MMCO Centricon (data not shown).

The fraction of extract from Tex6 retained by the 50-kDa MMCO Centricon was further fractionated by gel filtration chromatography using a Bio-Gel P-100 column. Sequential fractions were collected from the column and assayed in PMS medium for their effect on fungal growth, and fractions containing the AFGI were further purified by HIC (Fig. 4A). Fractions strongly inhibitory to fungal growth (fractions 7 and 8), along with adjacent fractions that showed either weak or no inhibitory activity in the assay, were subjected to SDS-PAGE, and the gel was developed by silver staining (Fig. 4B). Two protein bands, one at approximately 27 kDa and one at 22 kDa, were associated with the inhibitory activity. Assuming that the intensity of staining is indicative of protein concentration, the 27-kDa protein appears to be one associated with the maximum inhibition of fungal growth, as the band intensity correlates with inhibitory activity. When the sample was treated with β -mercaptoethanol and subjected to SDS-PAGE, the inhibitory protein migrated at 28 kDa (data not shown). Treatment of proteins with β -mercaptoethanol has been shown to result in a more accurate molecular mass estimation, because the protein assumes a less compact form.

Quantification of inhibitory activity. A dosage response curve for the growth inhibitor was demonstrated by diluting preparations from the Centricon containing the AFGI in PMS medium. After a logarithmic transformation of protein concentration, linear responses of inhibition were observed from 13 to 65 μ g of protein/ml for the extract of Tex6 (Fig. 5). Fungal growth was inhibited 50% by 26 μ g of protein/ml. A dosage response curve of the ABI was obtained by diluting anion exchange column fractions inhibitory to aflatoxin production in SLS medium. After a logarithmic transformation of aflatoxin concentration, a linear response between protein concentration and inhibition of aflatoxin biosynthesis was obtained for ABI (Fig. 6). Aflatoxin biosynthesis was

inhibited 50% at 75 μ g of protein/ml of this partially purified fraction. Both inhibitory activities were abolished by treatment with proteinase K (data not shown).

DISCUSSION

We report here the presence of two inhibitory activities in seed extracts of corn inbred Tex6. One compound inhibits fungal growth, while another inhibits aflatoxin biosynthesis, but has little effect on fungal growth. Preliminary characterization of the inhibitory compounds in corn inbred Tex6 indicated that these compounds are stable up to 40°C, insoluble in organic solvents, pre-precipitated by 50% saturated $(\text{NH}_4)_2\text{SO}_4$, and sensitive to proteinase K. These tests suggest that these inhibitors are proteins or protein-associated.

A

Fraction #	$(\text{NH}_4)_2\text{SO}_4$ gradient (M)	Fungal dry weight (mg)
1	pre-elution	1.0 ^b
2	1.50-1.35	1.4 ^a
3	1.30-0.90	1.2 ^{ab}
4	0.85-0.83	1.4 ^a
5	0.80-0.73	1.3 ^a
6	0.72-0.70	1.1 ^b
7	0.68-0.58	0.0 ^d
8	0.56-0.23	0.7 ^c
9	0.20-0.00	1.2 ^{ab}
Ctrl	N/A	1.3 ^a

Fig. 4. A, Hydrophobic interaction chromatography of the fungal growth inhibitor. Inhibition of fungal growth by each fraction is listed in the figure table. Numbers labeled with different letters are significantly different from each other ($P = 0.05$). The protein elution profile, monitored at 280 nm, is represented by the solid curve. Dotted lines in the graph represent the portion of the profile in each fraction assayed. **B,** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins contained in fractions from the hydrophobic interaction column in A. Thirty microliters of each fraction, as indicated by the numbers, was loaded in each lane. Molecular weight markers are indicated on the right side. Based on a correlation between the amount of protein and the amount of inhibitory activity, the putative growth inhibitory protein is hypothesized to be the 27-kDa protein (indicated by an arrow).

Fig. 3. Hydrophobic interaction chromatography of the inhibitory fractions collected from the DEAE anion exchange column as described in Figure 2. Inhibition of aflatoxin biosynthesis and fungal growth by each fraction is listed in the table. Numbers labeled with different letters are significantly different from each other ($P = 0.05$). The protein elution profile, monitored at 280 nm, is represented by the solid curve. Dotted lines in the graph represent the portion of the profile in each fraction assayed.

Using a laboratory assay that allowed us to reproducibly assay small sample volumes, we were able to follow the activity of the inhibitory compounds through a series of purification steps. Both of the inhibitory activities were resolved by HIC. The ABI compound appears to be more hydrophobic than the growth inhibitor, and of a larger molecular mass. Although this compound has not been characterized, it appears to have a molecular mass greater than 100 kDa, based on its retention by a Centricon filter with a MMCO of 100 kDa. Partially purified preparations of this compound were able to cause 50% inhibition of aflatoxin biosynthesis at 75 µg of protein/ml. This indicates that this compound is biologically active in physiological ranges and will likely show greater biological activity once it is more fully purified.

The fungal growth inhibitor appears to be smaller than the aflatoxin inhibitor. We have been able to associate the activity of this compound with one protein band on SDS-PAGE. The estimated molecular mass of the protein is 28 kDa. It is interesting that the AFGI was retained by a Centricon filter with a MMCO of 50 kDa. Possibly other compounds are associated with this compound in crude extracts, causing it to be retained on the filter.

Proteins involved in plant host defense against pathogens and insects have been shown to function by several different mecha-

nisms (1,31,32,35,37,42,43). Some are induced by the infection by pathogens, for example, the pathogenesis-related (PR) proteins (24,36). Some are preexisting proteins in intact plants (1,4,32,43). PR proteins and preexisting proteins or peptides with antimicrobial properties have been reported from corn kernels (11,13,16,37). The growth inhibitor detected in this paper significantly differs in size from PR-1, PR-2, and PR-4 type proteins. It does not have the chitinase activity associated with PR-3. The estimated molecular mass of 28 kDa is similar to that of PR-5 thaumatin-like proteins. One of these proteins, zeamatin, has a molecular mass of 22 kDa and has been isolated from corn seeds (37). While we observed a band at 22 kDa in the inhibitory fraction from Tex6, its concentration does not correlate with maximum inhibition of fungal growth. Since we did not characterize the protein, it is possible that the protein is zeamatin. We cannot rule out the possibility that the 28-kDa protein is also a thaumatin-like protein.

The AFGI also appears to differ from the preexisting basic peptide of 4,127 Da reported by Duvick et al. (16) and the ribosome-inactivating proteins that have a molecular mass of 30 kDa (38). It is not possible to compare our protein with the one found by Nagarajan and Bhat (25) based on the data presented in their paper.

Further characterization of the AFGI and ABI will allow us to determine if the active proteins we have isolated from Tex6 are similar to other antimicrobial compounds isolated from plants. There are two immediate uses of the compounds that inhibit aflatoxin production. The compounds can be used as a chemical marker (or the encoding genes as molecular markers) to screen for aflatoxin resistance in corn. Marker assistance will greatly reduce the time and labor, while improve the efficiency, in breeding. Also, once the gene that encodes this compound is cloned, it may be possible to engineer corn and other susceptible plants to produce the inhibitory compounds.

We may now be able to identify compounds responsible for resistance to aflatoxin accumulation. The resistance of Tex6 to ear rot and aflatoxin accumulation could result from a combination of ABI and AFGI. Because *A. flavus* has limited parasitic abilities and can grow and produce aflatoxin on injured kernels, some doubt has been expressed over the feasibility of identifying genotypes of corn with resistance to fungal growth or aflatoxin accumulation. The results of our study and those of others (5,8,10,33) now show host plant resistance is a feasible control strategy for the prevention of aflatoxin accumulation.

ACKNOWLEDGMENTS

This research was sponsored, in part, by USDA-SCA 58-6435-5-075. We thank DeKalb Plant Genetics for their support for research on the elimination of aflatoxin contamination in corn. We thank M. Daub and M. Carson (Department of Plant Pathology, North Carolina State University) for their valuable comments and suggestions in preparation of this manuscript. We also thank M. Carson for increasing the supply of Tex6.

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Fig. 5. Quantification of the activity of the growth inhibitors from the extracts of Tex6. Open square markers represent the dosage response using the extract from Tex6. Data points are the averages of four replicates. The dotted line indicates the protein concentrations that result in 50% inhibition of growth. The regression formula and the correlation coefficient (r) are indicated.

Fig. 6. Quantification of the activity of aflatoxin inhibitor on mycelial growth and aflatoxin production. The means of aflatoxin production are represented by square symbols and the means of mycelial growth are represented by bars. Error bars represent standard errors of four replicates. The dotted line indicates the protein concentration that inhibits aflatoxin production by 50%. The regression formula and its correlation coefficient (r) are indicated.

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