A Chitinase from Tex6 Maize Kernels Inhibits Growth of *Aspergillus flavus*

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ABSTRACT

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The maize inbred Tex6 has resistance to colonization and aflatoxin accumulation by *Aspergillus flavus*. A protein inhibitory to growth of *A. flavus* has been identified from aqueous extracts of mature Tex6 seeds. This study reports the purification of a chitinase associated with this inhibitory activity to electrophoretic homogeneity and the further characterization of its properties. The inhibitory protein, which has an M_r of

29,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is an endochitinase that is also capable of exochitinase activity. The enzyme has an optimal pH of 5.5 and a temperature optimum of 45°C. Chitinase activity in maize kernels peaked approximately 36 days after pollination. The Tex6 chitinase purified in this study is capable of inhibiting the growth of *A. flavus* by 50% at a concentration of 20 µg/ml. Our data indicate that chitinase activity in Tex6 kernels makes a major contribution to the antifungal activity in this maize genotype. Partial peptide sequence of the chitinase showed it to differ from previously reported chitinases.

The fungus *Aspergillus flavus* is responsible for both pre- and postharvest accumulation of aflatoxin in maize (*Zea mays*) (6,27). Concern about aflatoxin contamination is due to its potent carcinogenicity (5,8,27,32); aflatoxin B₁ is the most potent naturally occurring carcinogen known (32). It can induce liver tumors in rats at concentrations as low as 1×10^{-9} percent of the rat diet (36). Because of health concerns associated with aflatoxin contamination, the Food and Drug Administration regulates the concentration of aflatoxin allowed in feed and foods marketed within the United States. Internationally, other countries set their own limits for allowable aflatoxin contamination; often these levels are lower than acceptable by the United States. Aflatoxin contamination is clearly a hazard to animal health and an impediment to trade (24).

Aflatoxin contamination of maize in the United States is associated with above average temperature and below average rainfall (27,33). Cultural practices are effective in reducing aflatoxin contamination (4,27,33), but no currently available practice can prevent aflatoxin contamination in years favorable for the growth of the fungus. Plant resistance has proven successful for other diseases of maize and there is promise that this will be the case for reducing contamination of maize with aflatoxin. Breeding programs to identify and incorporate resistance to aflatoxin accumulation are ongoing, and a number of genotypes have been identified with resistance (2-5,7,13,30,34,35,38). Unfortunately, the incorporation of high resistance to either infection or aflatoxin accumulation in commercially valuable hybrids has not been successful.

Certainly the ability to identify markers associated with resistance would greatly facilitate the incorporation of resistance into desirable genotypes. To this end, research has focused on identifying compounds in maize seeds that inhibit fungal growth or the

accumulation of aflatoxin. The goal of this research is to identify markers that can be used to follow the segregation of resistance in a breeding population. Several compounds from seeds have been associated with resistance to infection by *A. flavus*. These include seed waxes (12,28), an α -amylase inhibitor (9), a trypsin inhibitor (6), ribosome inactivating proteins (11,25), zeamatin (11), and a β -1,3-glucanase (21). Proteins with chitinase activity have been shown to accumulate in maize seeds in response to infection by *A. flavus*, but a definitive role for these compounds in host resistance has not been determined (18,37).

Because maize inbred Tex6 has been shown to have resistance to infection and aflatoxin accumulation by *A. flavus* (4,5,13), we reasoned that this genotype would be a good candidate for studies directed at identifying compounds inhibitory to *A. flavus* and aflatoxin accumulation. We recently reported the isolation of two inhibitory compounds from Tex6; one that inhibits fungal growth and the other that inhibits aflatoxin formation (14). In this paper, we report further characterization of the compound shown to inhibit fungal growth and show that it is a chitinase that differs from previously reported chitinases.

MATERIALS AND METHODS

Tex6 extract preparation. Seed of Tex6, an inbred selfed from southern corn cv. Whitemaster (PI 401763) (5), was supplied by D. White at the University of Illinois at Urbana. Tex6 was grown and self-pollinated at the North Carolina Central Crops Research Station, Clayton, in the summers of 1998 and 1999. Ears were harvested at the developmental times indicated (between 26 and 40 days after pollination) and rapidly frozen in liquid nitrogen. The kernels were shelled onto dry ice and stored at -80°C until use. Seeds were ground at 4°C in a coffee grinder. The powder was suspended in 50 mM citrate buffer, pH 5.5, and stirred for 1 h (1 kg of powder to 3 liters of citrate buffer). This slurry was then filtered through cheesecloth and centrifuged at $6,000 \times g$ for 30 min. The resulting supernatant was filtered through Miracloth and designated Tex6 extract.

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Fungal inhibition bioassay. *A. flavus* strain 656-2 GAP 13-22 (14) was used in all bioassays. Inhibition of *A. flavus* by Tex6 was monitored according to an assay adapted from Huang et al. (14). Briefly, 10^6 conidia were incubated in 24-well microtiter plates in peptone mineral salts medium containing the sample to be tested (1,050 μ l total volume per well). The plates were incubated at 28°C for $\approx$ 90 h. The mycelial pads were harvested, washed with double-distilled H₂O (to remove salts and growth medium), dried, and weighed. Quantification of fungal inhibition was expressed relative to buffer controls.

Chitinase assays. *N*-acetylglucosamine, *N,N'*-diacetylchitobiose, and *N,N',N''*-triacetylchitotriose were obtained from Sigma Chemical, St. Louis. Chitinase activity was measured using high-performance liquid chromatography (HPLC) to quantify the concentration of chitin breakdown products. These breakdown products (*N*-acetylglucosamines) were separated on a carbohydrate analysis column (Waters Corp., Milford, MA) using a mobile phase of 75:25 acetonitrile/H₂O at a flow rate of 0.9 ml/min. Detection was determined at 210 nm. The retention times of monomer *N*-acetylglucosamine, dimer *N*-acetylglucosamine (*N,N'*-diacetylchitobiose), and trimer *N*-acetylglucosamine (*N,N',N''*-triacetylchitotriose) were 4.5, 8.0, and 14.2 min, respectively.

The chitinase activity assay was conducted by incubating colloidal chitin and Tex6 extract or column fractions in 50 mM citrate, pH 5.5, at 28°C. At selected time points, an aliquot was removed from the reaction vessel and injected directly onto the HPLC column. Integration of peak area associated with standard curves for the concentrations of mono, di, and tri *N*-acetylglucosamines gave a direct reading of chitinase activity. The temperature study was done at pH 5.5, and the pH study was done at 28°C.

Purification of Tex6 chitinase. All steps were performed at 4°C unless otherwise noted. Extracts from mature seed (described previously) were applied to a Sephadex G-100 GFC column (15 cm by 1.5 m) and eluted at a flow rate of 9 ml/h. The fractions that displayed antifungal activity were pooled and concentrated with a centrprep-10 unit (Millipore, Billerica, MA), and the pooled sample was subjected to chitin affinity chromatography. The affinity column (0.5 by 10 cm) contained 5 g of purified powder chitin from crab shells (Sigma Chemical) suspended in 50 mM citrate, pH 5.5. The column was washed with 50 mM citrate, pH 5.5, and eluted with 2 M NaCl by gravity flow. Active fractions from the affinity column were then added to a carboxy-methyl (CM) column (Bio-Rad Laboratories, Hercules, CA), washed with 50 mM citrate (pH 5.5), and eluted sequentially with 75 mM NaCl and 125 mM NaCl. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described previously (19). Gels were stained and visualized with Coomassie brilliant blue G-250.

RESULTS

Fractionation of the antifungal compound in Tex6 maize seeds. Previous research from our lab demonstrated that the fungal resistance of Tex6 maize is protein-mediated (14). This project sought to identify the protein or proteins responsible for the antifungal activity. We passed Tex6 seed extract through a Sephadex G-100 GFC column and assayed the resulting fractions for fungal inhibition as described previously. Figure 1A shows the results of the bioassay superimposed on the protein elution profile from the G-100 column. The growth of *A. flavus* was relatively unaffected by fractions from the G-100 column until fractions from the 25,000 to 35,000 *M_r* region were assayed (elution volume 600 to 800 ml). At this point, there was a dramatic decrease in fungal growth, which signifies the presence of inhibitory compounds. The antifungal activity coincided with a small but distinct peak of protein.

Among the previous reports of antifungal plant proteins with molecular weights of 25 to 35 kDa are β -1,3-glucanases (17,23) and chitinases (1,5,10). We tested Tex6 extract for chitinase ac-

tivity on a chitin agar plate and observed a small zone of clearing after 3 days incubation. Thus, the Tex6 extract contained an activity capable of degrading chitin (data not shown).

Evidence of both a chitin-degrading activity in the crude extract and an antifungal activity of $\approx$ 30 kDa suggested that the antifungal activity in Tex6 kernels might be a chitinase. Fractions from the G-100 column were assayed for chitinase activity by the chitin activity assay as described previously. Figure 1B shows a single large peak of chitinase activity with two shoulders. The chitinase activity was maximal in the fractions having the largest inhibitory effect on fungal growth. This circumstantial link between chitinase activity and antifungal activity prompted us to investigate whether the antifungal activity was due to a chitinase. Fractions from the G-100 column showing antifungal activity were pooled, concentrated, and applied to a chitin affinity column. The column was washed with 50 mM citrate and eluted with 2 M NaCl. Three fractions were then investigated: fraction 1, the active G-100 column fractions that had been shown to be antifungal; fraction 2, the material that was washed from the chitin affinity column with 50 mM citrate (a fraction in which the chitinase activity had presumably been removed); and fraction 3, the material eluted from the chitin affinity column with 2 M NaCl (a fraction which should contain chitinase activity). The 2 M NaCl eluant from the chitin affinity column was dialyzed 4,000:1 into 50 mM citrate, pH 5.5. The pooled antifungal fractions from the G-100 column

Fig. 1. Gel filtration chromatography of Tex6 seed extracts. Tex6 seed extracts were applied to a Sephadex G-100 column and the fractions were assayed for **A**, protein and inhibitory activity against *Aspergillus flavus* and **B**, chitinase activity and inhibitory activity against *A. flavus*.

were compared with the citrate wash and the NaCl wash from the chitin affinity column for chitinase activity, as determined by HPLC, and for fungal growth inhibition.

In the absence of seed extracts (control), *A. flavus* grew to a dry weight of 13.2 mg (Fig. 2A). In contrast, *A. flavus* growth was reduced to 0.1 mg when it was cultured on the pooled active fractions from the G-100 column that were applied to the chitin affinity column (fraction 1). The chitinase activity in fraction 1 was 1.3 µg/min/mg of protein. The citrate wash from the chitin affinity column (fraction 2), which had the majority of the chitinase activity removed (remaining chitinase activity was 0.1 µg/min/mg), supported fungal growth to 7.7 mg. This fraction retained 58% of its antifungal properties. Finally, the 2 M NaCl wash from the chitinase affinity column (Fraction 3) that contained a chitinase activity of 2,000 µg/min/mg reduced fungal growth to 0.3 mg of

Fig. 2. Analysis of Tex6 seed proteins after fractionation on a chitin affinity column. **A**, Growth of *Aspergillus flavus* in the absence of Tex6 seed proteins (control) and in the presence of three fractions subjected to chitin affinity chromatography. Fraction 1 is the pooled active fractions from the G-100 column described in Figure 1 that were applied to the chitin affinity column. High-performance liquid chromatography analysis showed chitinase activity to be 1.3 µg/min/mg. Fraction 2 is the wash from the chitin affinity column (chitinase activity was 0.1 µg/min/mg). Fraction 3 is the 2 M NaCl eluant from the chitin affinity column (chitinase activity was 2,000 µg/min/mg). In the growth assay, 700 µl of each fraction was assayed for antifungal activity according to the antifungal assay described in the text. **B**, A sodium dodecyl sulfate-polyacrylamide gel electrophoresis chromatogram of protein preparations subjected to chitin affinity chromatography. Lane 4 is a molecular size marker.

fungal dry weight. This fraction from the chitin affinity column reduced fungal growth by 98% and retained greater than 76% of the chitinase activity of fraction 1.

SDS-PAGE was used to visualize the partitioning of protein by the chitin affinity column (Fig. 2B). Coomassie blue staining of the gel showed a prominent band ($M_r \approx 29,000$) in the fraction eluted from the chitin affinity column with 2 M NaCl (fraction 3). This gel also showed that the majority of this putative chitinase band at 29 kDa had been removed from the G-100 fractions by the chitin affinity column, thus confirming the data obtained from HPLC analysis.

Purification of Tex6 chitinase. Chitinase activity has been reported in both developing and mature kernels as well as in germinating seeds of maize. We assayed Tex6 kernels for chitinase activity in immature seed between 26 and 40 days after pollination. The chitinase activity reached maximal levels approximately 36 days after pollination (Table 1). Therefore, we used 36-day postpollination kernels for subsequent chitinase purification. The chitin affinity column removed the majority of contaminating proteins leaving a single band at $M_r \approx 29,000$ in a SDS-PAGE gel. Upon close inspection of the SDS-PAGE gel, two bands of $M_r \approx 29,000$ were present (Fig. 3B, lane 3). The chitinase fractions

Days after pollination	Chitinase activity (µM/min/mg)
10	10.1 ± 0.2
28	11.1 ± 0.4
30	12.0 ± 0.5
33	13.3 ± 0.6
36	14.5 ± 0.3
39	13.5 ± 0.4
40	12.0 ± 0.8

Fig. 3. Separation of two putative chitinases from Tex6 by gel chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **A**, SDS-PAGE chromatography of the Tex6 protein fractions showing inhibitory activity. Lane 1, Tex6 seed extract; lane 2, active fractions from the G-100 column; lane 3, active fractions from the chitin affinity column; lane 4, molecular weight standards; lane 5, chitinase 1; and lane 6, chitinase 2. **B**, Elution of chitinase 1 and 2 from a carboxy-methyl (CM) column.

from the affinity column were then added to a Bio-Rad CM column washed sequentially with 75 mM NaCl and 125 mM NaCl. The chitinase that was released from the CM column with 75 mM NaCl was, by far, the more abundant of the two chitinases. This protein migrated as a single band through SDS-polyacrylamide gels and represented a 190-fold purification based on specific activity (Table 2). In immunoblots, it showed cross-reaction with antibodies derived against cucumber chitinase (data not shown). We will refer to this protein as chitinase 1. The chitinase released with the 125 mM NaCl wash migrated as a single band that represented a small portion of the total chitinase activity. Due to the small yield of the second chitinase, no further evaluations of its properties were possible.

Characterization of chitinase 1. The purified protein had an M_r of 29,000 as determined by SDS-PAGE. Its optimum pH was 5.5 with activity dropping off sharply after pH 6.0 (Fig. 4A). Its

TABLE 2. Purification of Tex6 chitinase 1 from maize kernels^a

	Total protein (mg)	Total activity (μM/min)	Specific activity (μM/min/mg)	Fold purification	% Yield
Tex6 extract	4,200	58,000	14	1	100
G-100	290	34,000	140	10	70
Affinity	9	22,000	2,500	180	40
CM	3	7,800	2,600	190	14

^a The chitinase was purified to electrophoretic homogeneity, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, resulting in a 190-fold enrichment as determined by specific activity.

Fig. 4. The effect of pH and temperature on Tex6 chitinase 1 activity. Initial reaction velocities were used to determine the rate of reaction. The appearance of chitin breakdown products (mono and di *N*-acetylglucosamines) was monitored by high-performance liquid chromatography analysis. **A**, Reaction velocities of Tex6 chitinase 1 at pH values ranging from 3 to 10. **B**, Reaction velocities of Tex6 chitinase 1 at temperatures from 4 to 65°C.

optimum temperature was 45°C (Fig. 4B). It is interesting to note that 50% of the maximal rate of enzymatic activity was still observed at 4°C.

HPLC has proven to be a powerful tool because it permits the monitoring of the appearance of chitin breakdown products (7,26). HPLC fractionation showed that the initial product of the reaction of Tex6 chitinase 1 was the dimer of *N*-acetylglucosamine (*N,N'*-diacetylchitobiose). Only as the concentration of dimer began to rise did we see the appearance of monomers of *N*-acetylglucosamine (Fig. 5). Late in the reaction, we also were able to detect trimers of *N*-acetylglucosamine as well as low concentrations of higher order oligomers of *N*-acetylglucosamines. These data suggest that Tex6 chitinase 1 is primarily an endochitinase although it is also capable of exochitinase activity.

Antifungal characterization of Tex6 chitinase 1. A dosage response curve was conducted using the purified Tex6 chitinase 1 to determine its antifungal characteristics against *A. flavus*. Mycelial dry weight was determined after the fungus was grown in the presence of chitinase 1 at concentrations from 5 to 2,000 μg/ml. These data showed that 50% growth inhibition of *A. flavus* was achieved at a protein concentration of 20 μg/ml (Fig. 6). Fungal growth was not detected at a protein concentration of 2,000 μg/ml.

Fig. 5. Release of chitin polymers after incubation of chitin at 28°C with 20 μl of Tex6 chitinase 1 at a concentration of 5 mg/ml. The initial product of the reaction is solely the dimer of *N*-acetylglucosamine (*N,N'*-diacetylchitobiose). As the concentration of dimer increases, the monomer of *N*-acetylglucosamine appears. Late in the reaction, trimer and higher order oligomers of *N*-acetylglucosamine appear.

Fig. 6. Growth of *Aspergillus flavus* in the presence of differing concentrations of Tex6 chitinase 1. The fungus was cultured at 28°C in differing concentrations of chitinase for 90 h.

Sequence analysis of Tex6 chitinase. Initial attempts at N-amino terminal sequencing suggested that the protein was N-blocked at the amino terminus. We were able to obtain internal amino acid sequence data from three regions of the protein. Figure 7 shows the amino acid sequence and the alignment of the Tex6 chitinase 1 compared with the maize seed chitinases A and B reported by Huynh et al. (15). Region one of Tex6 chitinase 1 showed 80% amino acid sequence identity to chitinase A and 93% sequence identity to chitinase B. Region two showed 91% sequence identity with both chitinases A and B. Region three of the Tex6 chitinase showed 91% sequence identity with chitinase A and 84% sequence identity with chitinase B (Fig. 7).

DISCUSSION

The focus of this study was to characterize the protein in Tex6 seed extracts that inhibits growth of *A. flavus*. A linkage was found between antifungal activity and chitinase activity in Tex6 kernels. When chitinase activity was removed from Tex6 seed extracts by a chitinase affinity column, the antifungal properties of the extract decreased dramatically. In contrast, fractions eluting from the affinity column with chitinase activity were inhibitory to fungal growth. Further, fungal inhibitory activity followed chitinase activity throughout the purification protocol, and at the final step of purification, it was associated with a single protein band found in an SDS gel. Partial peptide sequencing of the inhibitory protein showed sequence similarity to a known maize seed chitinase. These results indicate that chitinase activity is a primary component of the antifungal activity in Tex6 maize kernels.

The sequences of the peptides of the Tex6 chitinase place this chitinase in the *Chia4* gene family (20). It is most similar to chitinases A and B, two maize chitinases that have been shown to have antifungal activity (15). However, it is not identical to either of these two chitinases, and we hypothesize that these differences are important for the enhanced antifungal activity of the Tex6 chitinase. Minor modifications in sequence can greatly affect the activity of chitinases. Chitinases A and B, for example, show 91% nucleotide sequence identity and 87% amino acid identity, but have marked differences in their inhibitory activity to fungi (15). Both of these chitinases inhibited the growth of *Fusarium oxysporum*, *Alternaria solani*, and *Trichoderma reesei*, but required 10 times more chitinase B than chitinase A to cause the same degree of inhibition. Neither of these chitinases inhibited the growth of the plant pathogens *Gaeumannomyces graminis*, *Sclerotinia sclerotiorum*, or *Phytophthora infestans*.

The chitinase from Tex6 appears to be in a different gene family than two other chitinases (pCh2 and pCh11) isolated from maize leaves (37). These chitinases are members of the *Chia1* gene

family (20). Both of these chitinases are present in maize kernels and were induced in damaged and *A. flavus*-inoculated kernels. The greatest induction of chitinase pCh2 and pCh11 transcripts was found in the aleurone layers of the seed. Transcript abundance was much greater for pCh2 than for pCh11. Only low levels of transcripts were found in the embryo and no transcripts were observed in the endosperm. Transcripts of pCh2 were also induced in maize calli cultured with *A. flavus* (37), but no evidence was found correlating chitinase activity in maize calli with inhibition of *A. flavus* (21).

Ji et al. (18) also reported a chitinase induced in maize seeds in response to infection by *A. flavus*. They found a 29-kDa protein with chitinase activity that was induced in developing maize seeds by infection with *A. flavus*, but not after infection with *F. verticillioides* or drought stress. The biological activity of this chitinase against *A. flavus* was not tested.

Chitinase activity in Tex6 maize seeds appeared to increase as the seed matured up to 36 days after pollination. Interestingly, in a comparison between resistant and susceptible maize hybrids, Windham and Williams (34) found that the significant differences between resistant and susceptible hybrids were not detected until 42 days after mid silk, indicating that the factor(s) associated with kernel resistance increases with kernel maturity.

Although there are several reports in the literature of endochitinases from plants (10), we are unaware of any other chitinase that has been isolated from plants with inhibitory activity against *A. flavus*. The specificity of chitinases for fungal species has been reported (15,17,31), but the mechanism of this specificity remains unknown. We evaluated the Tex6 chitinase for activity against *F. verticillioides* and found no inhibitory activity at 1,275 µg/ml.

In contrast, the Tex6 chitinase shows efficacy against *A. flavus* at a concentration of 20 µg/ml. Huynh et al. (15) tested maize seed chitinases using a plate-clearing assay and found efficacy by adding 10 µg of purified protein in a volume of 10 µl. Our level of efficacy of 20 µg/ml equates to 0.2 µg per 10 µl, suggesting that the Tex6 chitinase has greater inhibitory properties against *A. flavus* than chitinase A and chitinase B against the fungi tested. Thus, the Tex6 chitinase appears to have high activity against *A. flavus* as well as specificity for *A. flavus*.

While our data support a role of a chitinase in the resistance of Tex6 to *A. flavus*, other factors likely work in concert with the chitinase to confer the degree of resistance observed in the field. Several reports have implicated the synergism of two or more pathogenesis-related proteins in causing a dramatic increase in the effective lethality to a potential pathogen (16,17,22,29,31). Mauch et al. (22) and Ji and Kuc (17) observed a synergistic effect through the combination of β-1,3-glucosidase and chitinase. Regardless, these compounds can serve as markers in breeding programs aimed at increasing the resistance of maize hybrids to aflatoxin contamination. Further, knowing the profile of inhibitory compounds present in different maize genotypes with resistance may allow the combination of different sources of resistance into a single genotype.

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Fig. 7. Comparison of partial peptide sequence of Tex6 chitinase 1 with maize chitinases A and B (15).

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