Resistance to *Aspergillus flavus* in Corn Kernels Is Associated with a 14-kDa Protein

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ABSTRACT

Chen, Z.-Y., Brown, R. L., Lax, A. R., Guo, B. Z., Cleveland, T. E., and Russin, J. S. 1998. Resistance to *Aspergillus flavus* in corn kernels is associated with a 14-kDa protein. Phytopathology 88:276-281.

Corn genotypes resistant or susceptible to *Aspergillus flavus* were extracted for protein analysis using a pH 2.8 buffer. The profile of protein extracts revealed that a 14-kDa protein is present in relatively high concentration in kernels of seven resistant corn genotypes, but is absent or present only in low concentration in kernels of six susceptible ones. The N-terminal sequence of this 14-kDa protein showed 100% homology to a corn trypsin inhibitor. The 14-kDa protein purified from resistant varieties also demonstrated in vitro inhibition of both trypsin activity and the growth of *A. flavus*. This is the first demonstration of antifungal activity of a corn 14-kDa trypsin inhibitor protein. The expression of this protein among tested genotypes may be related to their difference in resistance to *A. flavus* infection and subsequent aflatoxin contamination.

Additional keywords: antifungal protein.

Infection of corn (*Zea mays* L.) by *Aspergillus flavus* and the subsequent accumulation of aflatoxins is a sporadic problem in the midwestern United States, but a chronic problem in the southern states. Drought stress, high temperatures, and insect damage can exacerbate the problem (40). Aflatoxin contamination significantly reduces the value of grain as an animal feed and as an export commodity (27). *A. flavus* produces the chemically similar aflatoxins B_1 and B_2. Aflatoxin B_1 is the most potent naturally occurring carcinogenic substance known (35). Aflatoxins can cause mortality in or reduce the productivity of farm animals (34). Foodstuffs contaminated by aflatoxins also have been associated with increased incidence of liver cancer in humans (19,29).

In the past 2 decades, conventional breeding and screening programs have focused on identifying corn genotypes resistant to *A. flavus* infection and subsequent aflatoxin accumulation (24). Several genotypes have been identified as resistant to aflatoxin production in both field and laboratory studies (2,3,33,39). However, incorporating resistance from these sources into commercial hybrids requires identification and characterization of factors shown to be associated with resistance. This is very important because of numerous problems associated with routine evaluation of genotypes including variation in aflatoxin levels among samples, as well as evaluation procedures for kernel infection and aflatoxin level that are both time-consuming and costly. Screening for morphological or molecular markers associated with resistance would circumvent many of the problems currently faced.

Recent work by Guo et al. (14) indicated that wax and cutin in kernel pericarps was involved in resistance to aflatoxin accumulation in corn population GT-MAS:gk (39). Russin et al. (32) showed further that kernel wax from GT-MAS:gk was chemically different from, and present in greater amounts than, wax from kernels of susceptible hybrids. However, resistance in GT-MAS:gk still was evident when kernels were wounded prior to inoculation, which suggests that some subpericarp factors also may be involved in GT-MAS:gk's resistance (14). Further examination of kernel protein profiles revealed consistent differences in banding patterns between resistant and susceptible genotypes (13). Other evidence suggesting the possible involvement of kernel proteins in resistance came from a recent study by Huang et al. (20), in which they reported two kernel proteins from Tex-6 that may contribute to its resistance to aflatoxin accumulation. One protein, with a molecular mass of about 28 kDa, inhibited growth of *A. flavus* and, thus, the aflatoxin accumulation; the other protein, with a molecular mass greater than 100 kDa, inhibited aflatoxin formation with little effect on fungal growth (20). The objectives of the current study were to identify and characterize protein differences in dry kernels between corn genotypes resistant or susceptible to *A. flavus* infection and aflatoxin formation and to identify proteins associated with resistance. A preliminary report has been published (6).

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MATERIALS AND METHODS

Materials. Kernels of susceptible corn hybrid Pioneer 3154 were obtained from Pioneer Hi-Bred International, Inc. (Johnston, IA). Kernels of resistant population GT-MAS:gk were obtained from the United States Department of Agriculture-Agriculture Research Service (USDA-ARS), Insect Biology and Population Management Research Laboratory (Tifton, GA). Kernels of resistant inbreds T115, CI2, Tex-6, and MI82, as well as susceptible inbreds MO17, NC232, and B73 (3), were obtained from the Department of Plant Pathology, University of Illinois, Urbana. Kernels of resistant inbred Mp420 and susceptible inbred Va35 were obtained from USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS. Resistant inbred Yellow Creole and susceptible inbred Huffman were obtained from USDA-ARS, Southern Regional Research Center, New Orleans. *A. flavus* (strain AF13) was used in all studies. This strain produces large quantities of aflatoxins in culture and in developing cottonseed and corn kernels (11). The fungus was grown on V8 juice agar plates (5% V8 juice, vol/vol, pH 5.2; and 2% agar, wt/vol) at 30°C in the dark. Conidia from 7-day-old cultures suspended in deionized water were used as inoculum.

Protein extraction. Dry kernels (20 g) from each genotype were frozen in liquid nitrogen and ground to powder in an analytical mill (Tekmar A-10; Janke and Kunkel GmbH & Co., Staufen, Germany). Powdered kernels were extracted with 40 ml of chilled (0 to 4°C) phosphate-citrate buffer (84 mM citric acid and 30 mM Na_2HPO_4, pH 2.8) containing 14 mM 2-mercaptoethanol and 6 mM ascorbic acid (10). Extractions were carried out at 4°C for 60 min with shaking (200 rpm). The crude extracts were filtered through four layers of cheesecloth before centrifuging at 17,000 × *g* to remove debris. The supernatants were filtered through Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England), adjusted to pH 7.5 with 6 N NaOH, and centrifuged at 17,000 × *g* at 4°C for 20 min. Supernatants were subjected to ultrafiltration and exchange with phosphate buffer (10 mM, pH 7.5) using a 10-kDa cut-off membrane (Centriprep Concentrator; Amicon, Inc., Beverly, MA) and were concentrated from 30 ml to 4 ml. Protein concentration in each extract was assayed according to Bradford (1) using the Bio-Rad (Bio-Rad Laboratories, Hercules, CA) dye reagent and bovine serum albumin as a standard. The concentrated protein extracts then were stored at –80°C until further use.

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts was performed using 15% resolving gel with 4% stacking gel according to Laemmli (22). Low-range protein markers (Sigma Chemical Co., St. Louis) were used as molecular mass standards. The gels were electrophoresed (120 V, 1.5 h), stained with 0.125% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 h at room temperature, and destained in 50% methanol and 10% acetic acid. A 14-kDa polypeptide was chosen for further investigation because preliminary results indicated that the presence of this protein correlated with resistance in several corn genotypes.

Quantitation of the 14-kDa protein. The quantitation of the 14-kDa protein was performed using Bio-Rad's GS-700 Gel Densitometer (Bio-Rad Laboratories) and the associated "Molecular Analyst" software. The data presented in this report is the mean value of each genotype obtained in three experiments.

N-terminal amino acid sequencing. To identify and further characterize the 14-kDa protein, total proteins (200 µg) extracted from resistant genotypes GT-MAS:gk and Tex-6 were subjected to 15% SDS-PAGE. Thioglycolic acid was added to the upper buffer to 0.1% to prevent chemical blockage of the N-terminus. The proteins were then transferred to polyvinyllidene difluoride (PVDF) membrane, stained, and destained as described by Chen et al. (7). The protein band with a molecular mass of 14 kDa was cut out, and 50 pmoles of this protein were used for N-terminal amino acid microsequencing at Baylor Medical School (Houston).

Purification of the 14-kDa protein. A trypsin-Sepharose 4B affinity column (2 by 20 cm) was prepared using type III trypsin from bovine pancreas (Sigma Chemical Co.) and cyanogen bromide-activated Sepharose 4B (Sigma Chemical Co.) according to manufacturer's instructions. The crude protein extract obtained from 300 g of GT-MAS:gk kernels was precipitated overnight by adding ammonium sulfate to 80% saturation at 4°C. The precipitated protein was collected by centrifugation (12,000 × *g* for 20 min) and resuspended in 100 ml of column loading buffer (0.02 M $CaCl_2$; 0.05 M Tris-HCl, pH 8.2; and 1.0 M NaCl). The 14-kDa trypsin inhibitor protein was eluted as described (23). Fractions with absorbance at 280 nm greater than 0.1 were combined and concentrated. Glycine in the purified trypsin inhibitor sample was removed by exchange with 10 mM phosphate buffer (pH 7.5). Purity of proteins collected was assessed using SDS-PAGE.

Trypsin activity assay. Trypsin (type III, from bovine pancreas) was obtained from Sigma Chemical Co. Trypsin activity was assayed as follows: enzyme activity assays were conducted in 1.0-ml cuvettes. To each, 0.5 ml of 20 µg of trypsin per ml in 0.1 M Tris-HCl, pH 8.2, and 0.2 ml of 0.1 M Tris-HCl, pH 8.2, were added. The reaction was started by adding 0.3 ml of 1 mM α-*N*-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA) in 0.1 M Tris-HCl, pH 8.2. The change of absorbance was continuously monitored at 412 nm for 8 min at 25°C. When trypsin inhibitor was added into the assay, it was allowed to incubated with trypsin at 25°C for 10 min before adding substrate. The amount of buffer added was also adjusted so that the final volume is 1.0 ml. All assays were conducted three times.

Antifungal activity assays. Bioassays were performed in 96-well microtiter plates. A preliminary dose effect study had shown that incubation of *A. flavus* conidia with purified trypsin inhibitor at a concentration of 20 µM or higher inhibited both spore germination and hyphal growth. In this study, purified trypsin inhibitor protein was added to the wells at a final concentration of 40 µM in a final volume of 50 µl, which contained 0.1× potato dextrose broth (PDB) and 5×10^5 freshly harvested *A. flavus* conidia. Conidia were incubated for up to 12 h at 25°C. Negative controls were 10 mM phosphate buffer or heat-inactivated trypsin inhibitor. Since trypsin inhibitor is relatively heat-stable (5,37), the purified trypsin inhibitor protein along with proper amount of water was denatured at 100°C for 50 min, and precipitated protein was removed by centrifugation before adding to the wells of microtiter plates. Hyphal growth in the control containing heat-inactivated trypsin inhibitor was similar to that in the phosphate buffer control. Morphological changes of *A. flavus* spores and hyphae were examined periodically during incubation using light microscopy. Hyphal length and width were measured using an ocular micrometer. All bioassays were conducted three times.

RESULTS

Protein extraction and electrophoretic profile comparisons. Analysis of dry kernel proteins extracted from each of the 13 genotypes using 15% SDS-PAGE revealed some differences in protein patterns (Fig. 1). Notable among these was a 14-kDa protein band present at high levels in resistant genotypes, but absent or present at very low levels in susceptible genotypes (Fig. 1). Profile analysis using Bio-Rad's molecular analyst (Bio-Rad Laboratories) showed that this 14-kDa protein comprised from 13.5 to 22% of the total acid extractable protein in resistant genotypes, but only 0 to 6.4% in susceptible genotypes. The quantitation of the relative content of this 14-kDa protein in all 13 genotypes is summarized in Table 1.

N-terminal amino acid sequencing and homology comparison of the 14-kDa protein. The total acid extractable protein

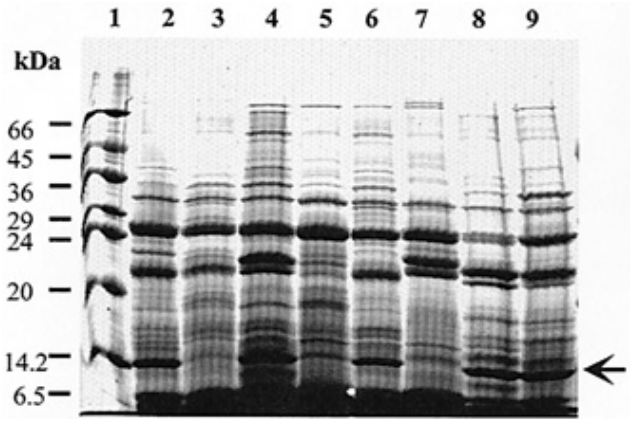

Fig. 1. A typical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts of resistant (R) and susceptible (S) corn kernels. Crude protein extract (10 µg) was loaded onto each lane of a 15% SDS-PAGE. After electrophoresis, gel was stained with Coomassie brilliant blue R-250. Lane 1, protein molecular weight markers; lanes 2 to 9, genotypes GT-MAS:gk (R), Pioneer 3154 (S), T115 (R), MO17 (S), CI2 (R), B73 (S), MI82 (R), and Tex-6 (R), respectively. The arrow indicates the position of the 14-kDa protein. The protein markers were albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14.2 kDa; and aprotinin, 6.5 kDa.

from GT-MAS:gk was resolved using SDS-PAGE, and the amino acid sequence of the N-terminal 26 residues of this 14-kDa protein was obtained through peptide sequencing (Fig. 2). Analysis of the N-terminal peptide sequence obtained from GT-MAS:gk revealed that it has 100% homology with the N-terminal sequence of corn 12-kDa trypsin/Hageman factor (factor XIIa) inhibitor protein (Fig. 2) (25,38). The 26 amino acid residues also showed significant homology with the N-terminal amino acid sequences of bifunctional trypsin/α-amylase inhibitor protein from finger millet (*Eleusine coracana*; 77%) (4), and barley (*Hv*-Ti1, *Hordeum vulgare*, 65%) (Fig. 2) (28). It has relatively low homology with α-amylase/trypsin inhibitor CM16 precursor from wheat (35%) (Fig. 2) (12). The first 10 amino acid residues of the 14-kDa protein isolated from Tex-6 were also determined, and the sequence was identical to that obtained from GT-MAS:gk.

Purification of the 14-kDa protein. Purification using a trypsin-Sepharose 4B affinity column yielded 4 mg of 14-kDa trypsin inhibitor protein from 300 g of GT-MAS:gk kernels. SDS-PAGE showed that the purified protein exhibited the same size as that in the crude protein extract (Fig. 3). Two minor polypeptides seen in the purified 14-kDa trypsin inhibitor sample (Fig. 3) are believed to be the 14-kDa protein digested by trypsin during the elution step of purification (9,23). The ability of the purified 14-kDa protein to inhibit trypsin was also examined. It was found that the activity of 10 µg of trypsin (23.8 kDa) was reduced by 50% after preincubation with 2.7 µg of trypsin inhibitor purified from GT-MAS:gk, and the activity was almost completely inhibited after preincubation with 5.5 µg of trypsin inhibitor.

Bioassays of the purified 14-kDa protein on fungal growth and development. Incubation of *A flavus* conidia suspension with 40 µM purified trypsin inhibitor not only caused significant spore rupture, but also reduced hyphal growth. The addition of 40 µM purified protein to conidia suspension resulted in rupture of about 45% of conidia within 1 h (Fig. 4). Initial spore rupture was observed within 5 min. Hyphal growth was also reduced by 23% in the presence of 40 µM trypsin inhibitor. After 12 h of incubation, hyphae developed from trypsin inhibitor treated conidia suspension were thicker (3.69 versus 2.42 µm on the average), shorter (59.4 versus 76.6 µm on the average), and exhibited an increased number of vacuoles compared with hyphae developed from untreated conidia (Fig. 5).

TABLE 1. The quantitation of the relative content of the 14-kDa protein band in the total protein extracts of different genotypes based upon analysis of 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles after staining with Coomassie brilliant blue R-250

Corn genotypes	Resistance to *Aspergillus flavus*	14-kDa protein content (%)
T115	R[a]	13.5
Mp420	R	14.1
CI2	R	15.3
Yellow Creole	R	16.6
Tex-6	R	17.0
GT-MAS:gk	R	19.0
MI82	R	22.0
B73	S	0.0
Va35	S	0.5
MO17	S	4.0
Pioneer 3154	S	4.1
NC232	S	6.2
Huffman	S	6.4

[a] R = resistant; S = susceptible.

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1    ---------------- -------------SA  2
2    MASSSSSSHRRLILA AAVLLSVLAAASASA 30
3    ---------------- -------------SV  2
4    -----MAFKYQLLLS AAVMLAILVATATSF 25
5    ----MASKSNCVLLL AAVLVSIFAAVAAIG 26

     GTSCVPGWAIPHNPL PSCRWYVTS------ 26
     GTSCVPGWAIPHNPL PSCRWYVTSRTCGIG 60
     GTSCIPGMAIPHNPL DSCRWYVAKRACGVG 32
     GDSCAPGDALPHNPL RACRTYVVSQICHQG 55
     NEDCTPWMSTLITPL PSCRDYVEQQACRIE 56
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Fig. 2. The N-terminal amino acid sequence of a 14-kDa protein from corn genotype GT-MAS:gk and its homology comparison with trypsin inhibitor proteins from different organisms. Lane 1, N-terminal amino acid sequence of the 14-kDa protein isolated from GT-MAS:gk; lane 2, 12-kDa trypsin inhibitor protein from corn (38); lane 3, α-amylase/trypsin inhibitor from finger millet (4); lane 4, trypsin inhibitor protein from barley (*Hv*-Ti1) (28); and lane 5, α-amylase/trypsin inhibitor CM16 precursor from wheat (12). The shaded blocks indicate identical amino acid residues.

DISCUSSION

Resistance to *A. flavus* in seven corn genotypes was associated with high levels of a 14-kDa protein in kernels, which suggests that this protein may be useful as a selectable marker in future efforts to screen or breed for resistance to this fungus. The N-terminal amino acid sequence of this 14-kDa protein has 100% homology with the N-terminal sequence of the 12-kDa trypsin inhibitor protein purified from corn kernels of Opaque-2 (Fig. 2), a genotype with demonstrated resistance to aflatoxin production (25, 36). Based on the deduced amino acid sequence of the cloned trypsin inhibitor cDNA (38), the mature polypeptide has a calculated molecular mass of 13,579 Da, which is very close to our estimation of 14 kDa based upon SDS-PAGE. The N-terminal amino acid sequence data, the purification of the 14-kDa protein using trypsin affinity column, and the inhibition of trypsin by this protein are strong evi-

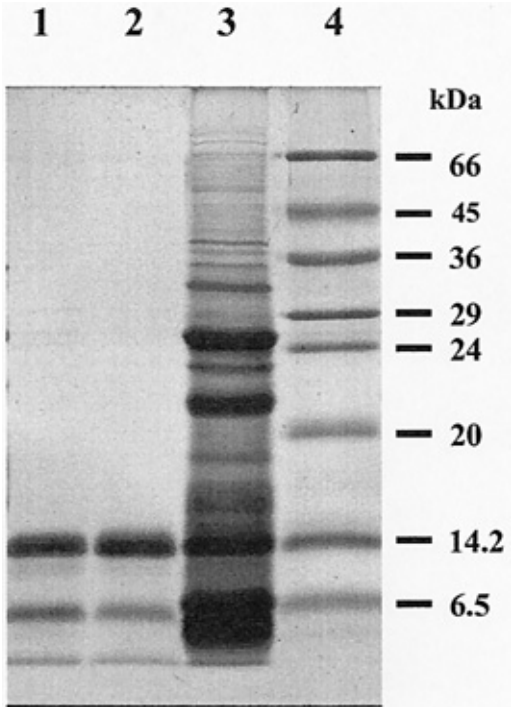

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the 14-kDa protein purified using affinity chromatography. Lanes 1 and 2, purified 14-kDa protein from GT-MAS:gk (1 µg/lane); lane 3, crude protein extract from GT-MAS:gk (10 µg); and lane 4, protein molecular weight markers. The two smaller protein bands in lanes 1 and 2 were believed to be trypsin-digested 14-kDa protein that occurred during elution of the column at pH 2.1 (9, 23). The gel was stained with Coomassie brilliant blue R-250, and protein markers were albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactalbumin, 14.2 kDa; and aprotinin, 6.5 kDa.

dence that the 14-kDa protein is a trypsin inhibitor. This protein also has been reported to be a specific inhibitor of activated Hageman Factor (factor XIIa) of the intrinsic blood-clotting process (18). It has little homology to the 7-kDa trypsin inhibitor (17) or the 22-kDa trypsin inhibitor protein (30) previously characterized from corn.

Recently, antifungal activities have been reported for trypsin inhibitor proteins from several organisms (8,37), including the 22-kDa trypsin inhibitor protein from corn (21). Our results show further that the 14-kDa protein in corn kernels also is antifungal against *A. flavus*. Early in 1973, Halim et al. (15,16) reported that endosperms of Opaque-2 corn exhibited much higher concentration of trypsin inhibitor than normal corn and showed retarded growth of *Fusarium moniliforme*, *Alternaria tenuis*, and *Periconia circinata* by more than 50% when the medium contained 200 to 400 µg of trypsin inhibitor per ml isolated from corn genotype Opaque-2. However, the molecular mass of the isolated trypsin inhibitor was not specified. Another interesting observation made by Nagarajan and Bhat (26) was that, among the seven hybrid varieties of corn they tested, Opaque-2 corn supported markedly low toxin after inoculation with *A. flavus*. A 5% NaCl extract of Opaque-2 corn resulted in almost total inhibition of the toxin production, and further fractionation of the NaCl extract revealed that the inhibitory factor was a protein of low molecular weight. The concentration of this protein was markedly higher in Opaque-2 than Deccan hybrid (26). It is possible that the low-molecular-weight inhibitory protein factor reported by Nagarajan and Bhat is the 14-kDa trypsin inhibitor. In the current study, we not only demonstrated that high levels of the 14-kDa protein are associated with seven corn genotypes resistant to aflatoxin contamination, but also demonstrated that the purified 14-kDa trypsin inhibitor from corn kernels is inhibitory to growth as well as spore germination of *A. flavus* when the fungus was treated with 40 µM trypsin inhibitor. Addition of commercial trypsin inhibitor purified from soybean as well as chitinase (EC 3.2.1.14) isolated from *Streptomyces griseus* at the same concentration and conditions did not cause inhibition of fungal growth or any observable morphological changes (data not shown). This suggested that changes in hyphal morphology as well as rupture of conidia caused by the 14-kDa protein were unique to this protein and may result from an effect on cell membrane permeability similar to those reported for

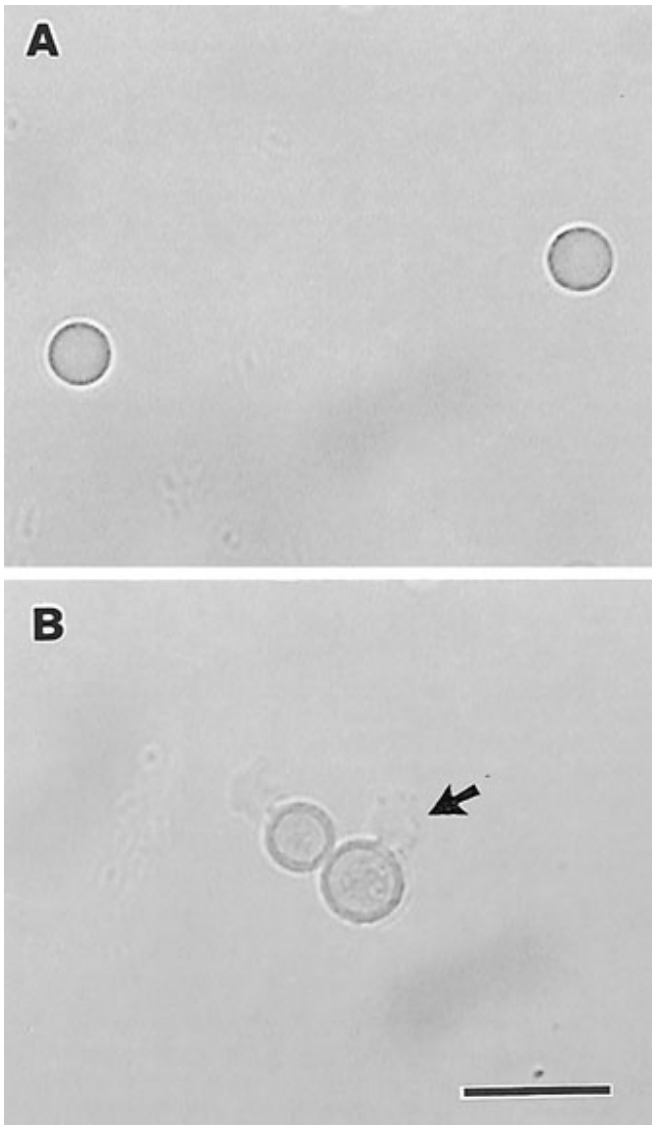

Fig. 4. Conidia of *Aspergillus flavus* in 0.1× potato dextrose broth after incubation at 25°C for 1 h with **A,** 10 mM phosphate buffer or **B,** 40 µM 14-kDa trypsin inhibitor protein purified from kernels of corn genotype GT-MAS:gk. Bar represents 10 µm. Arrow indicates discharged content of spores.

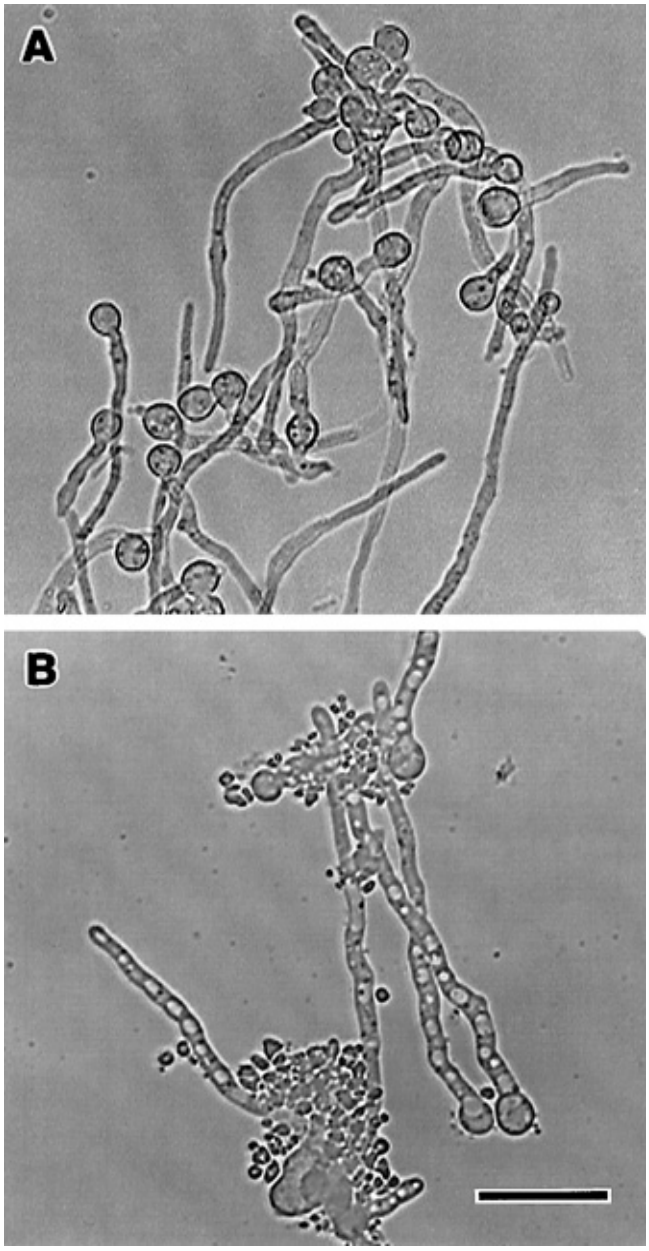

Fig. 5. Hyphae of *Aspergillus flavus* in 0.1× potato dextrose broth after incubation at 25°C for 12 h with **A,** 10 mM phosphate buffer or **B,** 40 µM purified 14-kDa trypsin inhibitor protein from kernels of corn genotype GT-MAS:gk. Bar represents 20 µm.

zeamatin (31) and thionins (37). A similar study by Huang et al. (20) found two proteaceous fractions inhibitory to aflatoxin formation from resistant genotype Tex-6. However, only two corn genotypes were compared, and no further characterization of these two fractions were made besides the estimation of molecular masses.

Although the concentration of the 14-kDa trypsin inhibitor required to demonstrate antifungal activity was relatively high in our assays, it is possible that the 14-kDa protein from corn kernels may not act alone, but rather in concert with other proteins. It was reported that the protein concentration required for 50% growth inhibition (IC_{50}) for barley trypsin inhibitor *Hv*-Ti1, which has significant homology with corn 14-kDa trypsin inhibitor (Fig. 2), ranged from 280 to >1,000 μg/ml on the four fungi tested (37). However, addition of only 10 μg of *Hv*-Ti1 per ml increased the antifungal activity of α-purothionin up to 20-fold, depending on the fungal species used (37). The level of antifungal activity may also be affected by other factors, such as salt concentrations (31,37). It is possible that the concentration of the 14-kDa protein required to show antifungal effects may be further reduced under slightly modified experimental conditions. In recent studies, reduced spore germination and hyphal growth were readily observed in conidia suspensions of *A. flavus* and other fungi treated with 7.2 μM trypsin inhibitor overexpressed from *Escherichia coli* (Z.-Y. Chen, R. L. Brown, A. R. Lax, T. E. Cleveland, and J. S. Russin, *unpublished data*).

In conclusion, we report that a 14-kDa trypsin inhibitor protein is associated with resistance to *A. flavus* in seven corn genotypes, and that incubation of this protein at 40 μM with *A. flavus* caused spore rupture and abnormal hyphal development. Future studies will examine the antifungal mechanism of this 14-kDa trypsin inhibitor protein and the feasibility of using it as a marker to develop commercially important corn genotypes resistant to *A. flavus* infection and aflatoxin contamination.

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