Inhibition of *Aspergillus flavus* Growth by Fractions of Salt-Extracted Proteins from Maize Kernel

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In vitro studies were used to determine the nature of fungal growth inhibition (*Aspergillus flavus*) by buffered saline-soluble kernel proteins in highly susceptible (Huffman) and highly resistant (Yellow Creole) open-pollenated varieties of maize. For both varieties, the isocratic protein fractions from preparative anion-exchange chromatography showed sporadic fungistatic effects in solid agar media. In both solid and liquid media, chromatographic fractions immediately following isocratic elution showed fungal growth inhibition only in the resistant variety. Assessment of lectin activity in pooled fractions from anion-exchange chromatography showed correlation with fungal growth inhibition for Yellow Creole in solid media but not in liquid media. For Huffman, lectin activity occurred primarily in the first two gradient-eluted fractions that were pooled after anion-exchange chromatography; the latter showed retardation of fungal growth in neither liquid nor solid media.

Aflatoxin contamination of hybrid maize as a major problem in the southeastern United States was shown to exist in preharvest and stored grain (Marsh and Payne, 1984; Stoloff, 1976). Most of the hybrids introduced in the mid-1940s were developed at a fast rate under controlled conditions. In contrast, the open-pollenated varieties used during the prehybrid era were hand-harvested, and selections were made over many generations. A recent study showed that select hybrids of maize tested at several locations in the southeast had lower aflatoxin levels than the open-pollenated varieties (Zuber et al., 1983); it was noted, however, that a few of the open-pollenated varieties tested were comparable to hybrids in aflatoxin contamination, indicating different levels of resistance to *Aspergillus flavus* infection. The complex relationship between *A. flavus* infection and aflatoxin formation is an area of intense research. Delineation of causal effects among varieties or hybrids is difficult to assess because multiple parameters such as insect damage and agronomic conditions are integral factors (Fennell et al., 1975; Hesseltine et al., 1976). Intrinsic factors and latent mechanisms that can affect fungal growth range from simple organic compounds to macromolecules such as complex polypeptides and polyphenols (Kommedahl and Williams, 1983; Roberts and Selitrennikoff, 1986).

In this paper, we describe the response of isolated protein fractions to mycelial growth and sporulation of *A. flavus* in vitro. We chose kernels from open-pollenated varieties with extremes in aflatoxin contamination due to *A. flavus* infection, namely Yellow Creole (highly resistant) and Huffman (highly susceptible). These two varieties are among several that were field tested by Zuber et al. (1983). Correlation of fungal growth inhibition with lectin activity in pooled isolated protein fractions from the two varieties was also assessed.

MATERIALS AND METHODS

Kernel Milling and Protein Extraction. Three hundred gram kernel samples of each variety, Yellow Creole (12.1% protein) and Huffman (10.2% protein), were milled on a standard Wiley Mill with a 40-mesh screen. Each meal was then extracted with 4 L of hexane to remove lipids and subsequently air-dried. Twenty-gram samples of the dried meals were extracted according to Landry and Moureaux (1970) with three 200-mL portions of phosphate buffer in saline (0.0009 M $NaH_2PO_{41}\cdot H_2O$, 0.0096 M Na_2HPO_4, 0.5 M NaCl, pH 7.8) on a Tekamr homogenizer at 25 °C. The homogenates were clarified by centrifugation at 18000*g* for 30 min at 25 °C. The final supernants (550-mL each) were dialyzed in 3.5-kDa cutoff bags against three 8-L volumes of deionized water for 3 days in a cold room. The whole content in each dialysis bag was freeze-dried and stored at −20 °C pending chromatographic procedures and bioassays. Dry weights corresponded to 8.3% for Yellow Creole and 9.8% for Huffman of total protein in dry kernels.

Chromatography on Ion-Exchange Cellulose. Eighty milligrams of protein from the two varieties was dissolved into 250 mL of phosphate buffer, pH 7.8, ionic strength 0.03, dialyzed in the same buffer overnight and absorbed on 4 g of a DEAE-cellulose (BIO-RAD cellex D) bed measuring 1.5 × 30 cm. Following isocratic elution in 5-mL fractions at 1.6 mL/min, the remaining protein was eluted with a 500-mL linear sodium chloride gradient ranging from 0.0 to 0.6 M NaCl in the above phosphate buffer at the same volume and rate (Dechary et al., 1961). These experiments were carried out at 25 °C. Each fraction was analyzed for protein by the Lowry method using BSA as a standard (Lowry et al., 1951).

Hemagglutination Assays. Human red blood cells (RBC) of the A^+ group were obtained from a local blood bank. The cells were washed several times with phosphate-buffered saline (Hierholzer and Suggs, 1969) and centrifuged each time at 2000*g* for 30 min at 15 °C. With 96-well microtitration plates (Cook's Engineering Corp., Alexandria, VA), hemagglutination assays were performed according to Prigent and Bourrillion (1976) by mixing samples with 10% RBC suspensions. After agitation, the suspension was allowed to settle for several hours at room temperature, during which time relative titers were assessed visually. The samples tested are described in the legend of Figure 3.

Bioassays of Fungal Growth Inhibition. *A. flavus* spores (SRRC 1000-A) were maintained on potato dextrose agar (PDA) slabs (Difco Laboratories, Inc., 1953); spore suspensions (3.2×10^7/mL of distilled water) were prepared from 10-day cultures of *A. flavus* growing on PDA. To observe effects by select protein fractions on fungal growth in solid medium, Corning polystyrene tissue cluster plates (24-well, 6-mm diameter) were filled with 2.0 mL of 2.0% PDA. For assays, each tissue cluster plate well was inoculated with 0.2 mL of test sample and with 0.05 mL of the *A. flavus* spore suspension. The inoculated media were at 25 °C without shaking up to 8 days and monitored for inhibition with a zoom stereo microscope.

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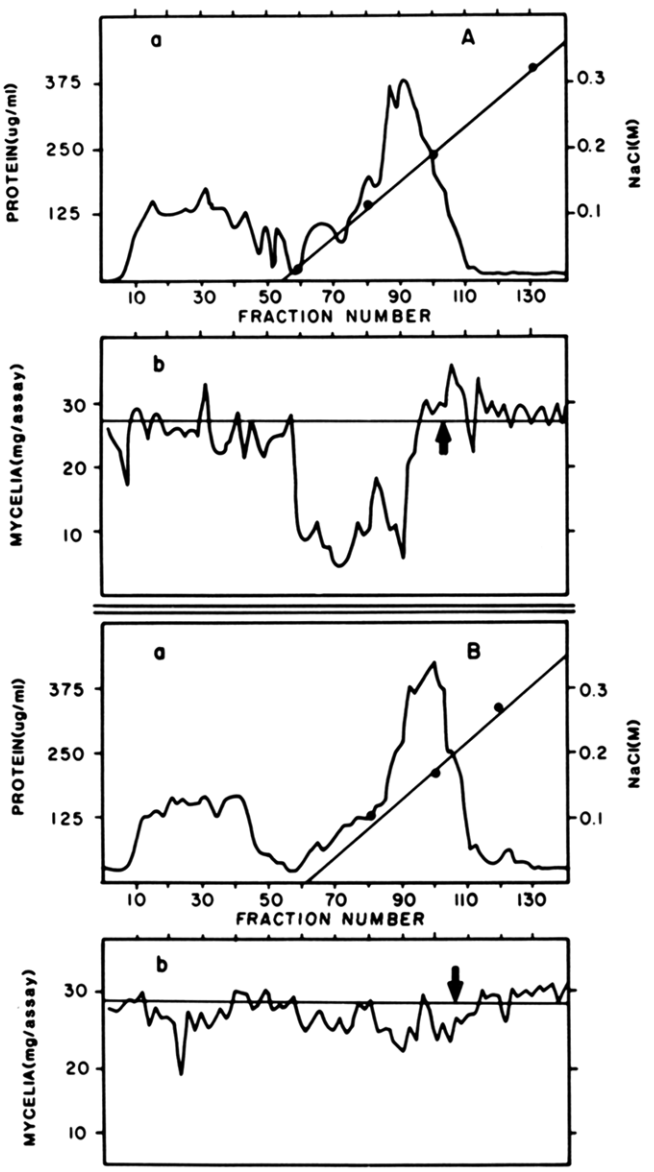

Figure 1. Chromatographic profiles of buffered saline-soluble proteins (A-a; B-a) and gravimetric assays of fungal growth inhibition (A-b; B-b) in liquid medium. A corresponds to Yellow Creole and B refers to Huffman maize varieties. The horizontal lines designated by arrows in b show mean mycelial weights of several controls. Closed circles in the chromatographic profiles refer to gradient salt measurements. See text for procedural details.

To assess gravimetric measurements on mycelial growth inhibition, the same volumes of test samples (and several controls) and *A. flavus* inoculum as in the above experiments were applied to 2.0 mL of liquid A & M medium (Adye and Mateles, 1964) in tissue cluster plates. After 7 days at 25 °C, the mycelia were collected into tared 2.0-mL Beckman microfuge tubes, dried at 100 °C for 2 days, and weighed directly.

Mycotoxins have to be handled as very toxic substances. Official procedures and guidelines have been established to perform experimental manipulations (Stoloff and Scott, 1984).

RESULTS AND DISCUSSION

Typical chromatographic profiles of proteins from Yellow Creole (A-a) and Huffman (B-a) and the effects of isolated fractions on *A. flavus* growth (A-b, B-b) in liquid A & M medium are shown in Figure 1. Preparative chromatography showed that at least one-third of the

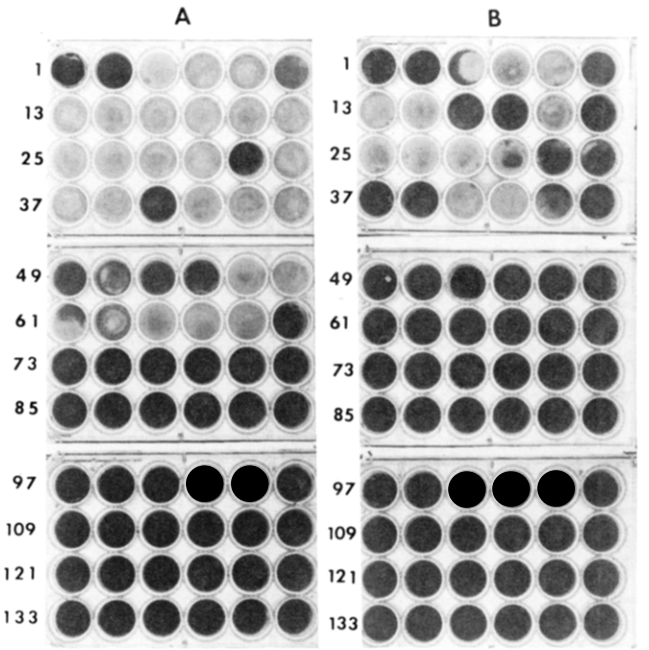

Figure 2. Antifungal effects on *A. flavus* inoculum in solid agar of every other chromatographic fraction described in Figure 1: A, Yellow Creole; B, Huffman. The last three wells in both A and B correspond to the controls. Dark areas denote massive mycelial growth and sporulation. The tissue plates were photographed 7 days after fungal inoculation.

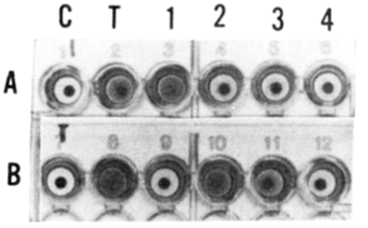

Figure 3. Hemagglutination assays of pooled fractions after chromatographic separation: A, Yellow Creole; B, Huffman; C, control or blank; T, total protein extracts before chromatography. Numbers 1–4 correspond to pooled fractions 1–58, 59–85, 86–95, and 96–125, respectively, described in Figure 1. Each test employed 200 μg of protein and 0.1 mL of 10% RBC of the A^+ group.

protein applied was eluted in the isocratic fractions (tubes 1–58) in both varieties; the remaining proteins were completely eluted after the gradient reached 0.25 M NaCl. To assess inhibitory properties of the fractions to *A. flavus* inoculum in A & M medium, every other fraction was inoculated with the spore suspension and allowed to incubate at 25 °C for 7 days. Fungal growth inhibition was observed primarily in fractions 58–88 (A-b) from Yellow Creole.

Results from experiments to test inhibition of fungal growth by these fractions in solid agar medium are shown in Figure 2. The data showed sporadic inhibition by some of the fractions in the isocratic eluant (tubes 1–47) for both Yellow Creole (A) and Huffman (B). The major difference between the two varieties is that several fractions at initial salt elution (tubes 57–69) from Yellow Creole showed a distinct trend of fungal growth inhibition; this was not observed for any of the comparable fractions in Huffman.

The correlation between inhibition of fungal growth and lectin activity (measured as hemagglutination of red blood cells) was assessed (Figure 3). The four pooled fractions from the chromatographic separations, designated 1–4, were dialyzed against distilled water, freeze-dried, and reconstituted in phosphate-buffered saline for analysis.

With equal quantities of protein applied to each well, only the total buffered saline-soluble fraction (T) and the isocratic pool (1) for Yellow Creole (A) showed hemagglutination. For Huffman (B), major hemagglutination activity occurred in pooled fraction 2 followed by weaker activity in pooled fraction 3. Results indicated that at least 200 μg of protein was required for visible agglutination. These results showed correlation between antifungal effects and lectin activity for Yellow Creole in solid agar assays; in A & M medium, however, the major antifungal effects occurred in individual fractions corresponding to pooled fraction 2. In Huffman, no correlation between lectin activity and fungal growth inhibition (solid or liquid media) was observed.

The specific activities of lectins in separated fractions of maize proteins were reported recently (Newburg and Concon, 1985). Although some activity was detected in saline-soluble extracts, most of the total activity was concentrated in the alkali-soluble glutelins. Vast literature on plant lectins have described their structures, sugar specificities, and practical uses as analytical tools; however, their physiological function that might benefit the general health of plants remains unclear (Etzlor, 1981; Brambl and Gade, 1985). One study postulated that wheat germ agglutinin inhibits chitin synthesis and spore germination in certain fungi (Mirelman et al., 1975).

These preliminary results clearly demonstrate inhibition of normal metabolic pathways in *A. flavus* growth induced by polypeptides from maize and might explain differences in aflatoxin levels observed under field conditions. Physicochemical properties such as molecular size, antigenic/electrophoretic behavior, and amino acid data of isolated protein are currently under investigation.

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Inhibition of Lipid Peroxidation by Ellagic Acid

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Ellagic acid, a common plant phenol, was found to be an effective inhibitor of in vitro lipid peroxidation by the erythrocyte ghost and microsome test systems. The structure–activity relationship of ellagic acid and two of its derivatives has been carried out, and it was suggested that ellagic acid was the most potent inhibitor of the perferryl-dependent initiation step of NADPH-dependent microsomal lipid peroxidation. Ellagic acid also strongly inhibited lipid peroxidation induced by Adriamycin, but the two ellagic acid derivatives were much less effective. This difference was true of all NADPH-dependent microsomal lipid peroxidations.

Oxygen species such as hydroxy radicals, superoxide anion radicals, and singlet oxygens are proposed to be agents that attack polyunsaturated fatty acids in cell membranes and give rise to lipid peroxidation. Several reports have suggested that lipid peroxidation may lead to destabilization and disintegration of cell membranes, to liver injury and other diseases, and finally, to aging and susceptibility to cancer (Player, 1982).

Recently, much attention has focused on cellular protective systems against damages caused by oxygen radicals including enzymes, such as superoxide dismutase, GSH-peroxidase, GSH-transferase, and catalase, as well as nonenzymatic protection of polyunsaturated fatty acid by endogenous antioxidants like α-tocopherol, ascorbic acid, β-carotene, and uric acid (Ames et al., 1981). In addition, several antioxidants have been reported to play an im-

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