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RAPID COMMUNICATIONS

Antifungal and Electrophoretic Properties of Isolated Protein Fractions from Corn Kernels

Keywords: *Aflatoxins; antifungal factors; Aspergillus flavus; polypeptides*

INTRODUCTION

Aflatoxin contamination of corn under both preharvest field conditions and stored grain is a major problem in the southeastern United States. The complex relationship between *Aspergillus 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. Intrinsic factors and latent mechanisms in plants that can affect fungal growth range from simple organic compounds to macromolecules such as complex polypeptides and polyphenols (Caruso et al., 1996; Kommedahl and Williams, 1983; Roberts and Selitrennikoff, 1986; Vigers et al., 1991).

In this brief study, we describe the response of isolated protein fractions from a resistant corn variety to mycelial growth and sporulation of *A. flavus* in vitro as a follow-up of a previous study (Neucere and Zeringue, 1987). The molecular sizes of the proteins in the bioactive fractions were characterized by electrophoresis.

EXPERIMENTAL PROCEDURES

Sample Preparation and Protein Extraction. A 300-g kernel sample of the corn variety Yellow Creole was milled on a standard Wiley Mill with a 40-mesh screen. The meal was then extracted with 4 L of hexane to remove lipids and subsequently air-dried. A 20-g sample of the dried meal was extracted according to Landry and Moureaux (1980) with three 200-mL portions of phosphate buffer in saline (0.0009 M $NaH_2PO_4 \cdot H_2O$, 0.0096 M Na_2HPO_4, 0.5 M NaCL, pH 7.8) on a Tekmar homogenizer at 25 °C. The homogenates were clarified by centrifugation at 18000*g* for 30 min at 25 °C. The final supernant (550 mL) was dialyzed in a 3.5-kD cutoff bag against three 8-L volumes of deionized water for 3 days in a cold room. The whole content of crude protein in the dialysis bag was freeze-dried and stored at −20 °C pending chromatographic separation, bioassays, and electrophoresis.

Analytical Procedures. DEAE-Spherodex column chromatography. Protein (160 mg) (BCA procedure) in 20 mL of 10 mM sodium phosphate buffer, pH 7.8, was applied to a DEAE-Spherodex (IBF Biotechnics) column as described earlier by Keller et al. (1993). The first 15 fractions were eluted with the lower ionic strength phosphate buffer. A linear gradient of 0−0.5 M KCl in the column buffer was then applied to elute fractions 16−32. Open circles correspond to antifungal activity.

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 from the chromatographic process and with 0.05 mL of the *A. flavus* spore suspension.

Electrophoresis. SDS-PAGE was conducted according to Laemmli (1970). The 20 × 20 cm^2 gels contained 5% (w/v) acrylamide in the stacking medium, and 12% (w/v) acrylamide in the running gel. Rainbow markers (Amersham, Inc.) ranging from 14.0 to 200 kD were employed. Each slot was filled with 100 μL of test sample. Electrophoresis was conducted at constant current of 50 mA per slab for approximately 5 h at 10 °C. Gels were stained in 0.125% Coomassie Blue R (Sigma) in methanol:acetic acid:water (4:2:5, v:v:v) and destained with the same solvent system. Protein concentrations were determined according to the BCA method (Pierce).

RESULTS AND DISCUSSION

The chromatographic profile of the salt soluble proteins from resistant corn is shown in Figure 1. The isocratic eluate showed two distinct peaks, and the salt-eluted fractions, one major peak. Five fractions (5, 6, 9, 10, and 15) from isocratic elution showed inhibition

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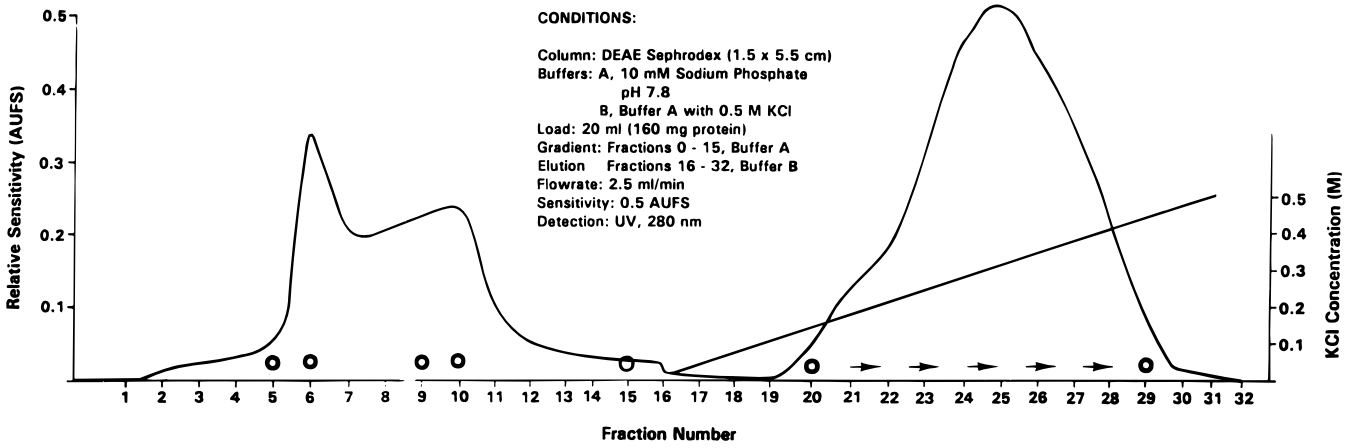

Figure 1. Elution profile of protein fractions from resistant corn off ion-exchange Spherodex. The first 15 fractions (2.5 mL) correspond to isocratic elution, and a gradient was applied starting at tube 16. The open circles correspond to fractions that showed antifungal properties as described in Figure 2.

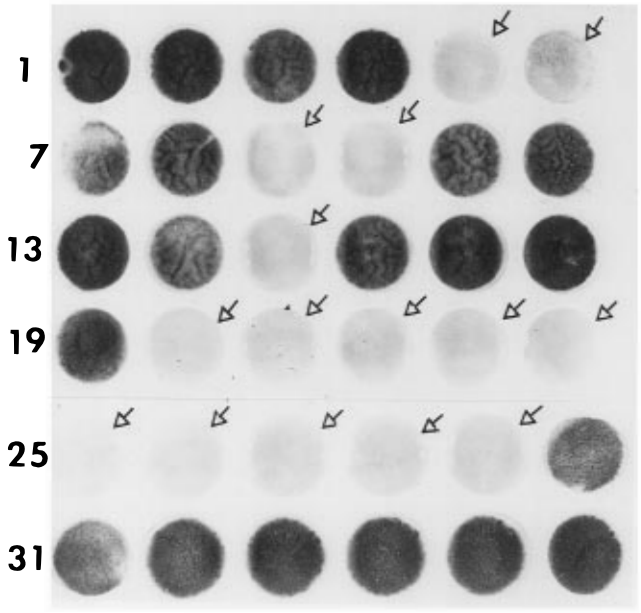

Figure 2. Antifungal effects of ion-exchange chromatographic fractions of corn proteins on *A. flavus* inoculum in solid agar. For assays, each tissue cluster plate well was inoculated with 0.2 mL of test sample and with 0.05 mL of the *A. flavus* spore suspension. The inoculated media were at 25 °C without shaking for 8 days. The clear areas (arrows) denote zones of fungal-growth inhibition. The last four wells correspond to 0.05 mL of spores alone.

of fungal growth, and essentially all of the fractions following gradient elution inhibited fungal growth in vitro.

Results from experiments to test inhibition of fungal growth by the isolated fractions in solid agar medium are shown in Figure 2. A distinct pattern of fungal growth inhibition by qualitative bioassay was observed for essentially all of the salt-eluted fractions numbered 20−29. Only five factions (5, 6, 9, 10, and 15) from isocratic elution showed bioactivity. The experiments showed that primary inhibition was directly related to the high protein content of the salt-eluted fractions.

Assessment of molecular size by SDS-PAGE of the salt-eluted fractions is shown in Figure 3. Fractions 25−28 showed the highest intensities of staining and the most complex arrays of protein banding. In these samples, a cluster of protein bands appeared in the 40−75-kD range. One protein component of approximately 16 kD occurred in all of the samples at varying concentrations. Some of the components appeared to migrate with the marker dye (less than 14 kD), indicating a group of low molecular weight proteins that might interact with the fungal spores. It should be noted here that fractions 20 and 21 were not included in the electrophoretic assay because of limited sample material.

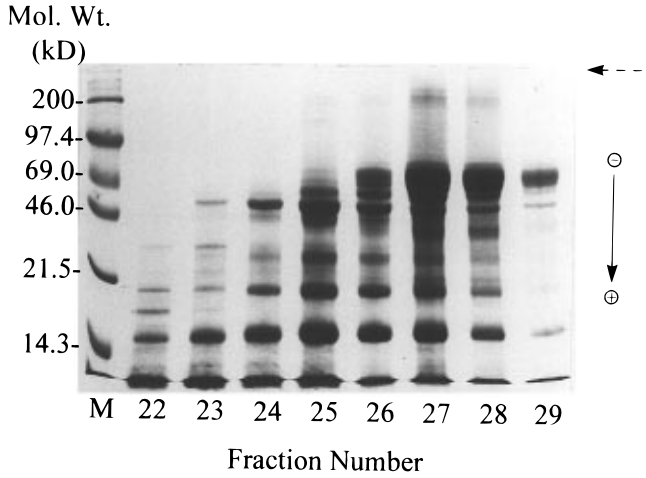

Figure 3. SDS-polyacrylamide gel stained with Coomassie Blue. Molecular masses in kilodaltons (kD) are indicated on the left. Individual protein bands were not completely resolved, and the intensity of staining appeared to be proportional to protein content. The band concentration was highly significant in the 46−85-kD region. The dashed arrow corresponds to the origin of electrophoresis.

The relationship between these partially isolated proteins and their actual participation in fungal growth inhibition and aflatoxin biosynthesis is not clear. To better understand the nature of these phenomena, however, optimal potency of isolated protein bands, the role of synergism among proteins, the complexity of carbohydrates involved, and examination of other genotypes will require further studies. For the future it will be interesting to determine whether antifungal proteins in corn do, indeed, play a significant role in defense against fungi and aflatoxin production in the field and whether or not they are important factors among the major cereal crops.

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