Electrophoretic Analysis of Cationic Proteins Extracted from Aflatoxin-Resistant/Susceptible Varieties of Corn

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The cationic proteins from two varieties of corn, *Zea mays* L., that are resistant (Yellow Creole) and susceptible (Huffman) to aflatoxin contamination were characterized by electrophoresis. Under native conditions, cathodic electrophoresis showed a cluster of proteins at R_f = 5.0–6.5 in Yellow Creole that was not evident in Huffman. SDS–PAGE showed approximately 12 bands that ranged between 14 and 43 kDa in both varieties. The profiles were different, both qualitatively and quantitatively. The major proteins in Yellow Creole clustered around 20 kDa on the gel and those in Huffman around 25 kDa. Qualitative variations of proteins by two-dimensional electrophoresis were evident, especially within the pH range 6.7–8.0 and around 27 and 92 kDa. The results are discussed in connection with the possible role of polypeptides as antifungal agents in species of corn.

In a previous study (Neucere and Zeringue, 1987), we showed that a large number of isocratic protein fractions recovered from anion-exchange cellulose chromatography from both *Aspergillus flavus* resistant and susceptible varieites of corn, *Zea mays* L., inhibited germination of fungal spores. Destructive activities on microbial cells by complex proteins associated with carbohydrates and lipids from both plant and animal tissues have been reported extensively in recent years (Albersheim and Anderson-Prouty, 1975; Barkai-Colan et al., 1978; Coleman and Robert, 1982; Olson et al. 1977; Roberts and Selitrennikoff, 1986). Many of these defense compounds exert lethal effects on biological systems by destroying enzyme activity, distorting cell membranes, and other unknown mechanisms of interactions.

This paper describes electrophoretic profiles of the cationic proteins from highly resistant (Yellow Creole) and highly susceptible (Huffman-white) genotypes of corn to *A. flavus* infection that were investigated earlier (Neucere and Zeringue, 1987; Zuber et al., 1983).

MATERIALS AND METHODS

Protein Extraction and Ion-Exchange Chromatography. Three hundred gram kernal samples of each variety 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 then subsequently air-dried. Twenty-gram samples of the dried meals were extracted according to the method of Landry and Moureaux (1980) with three 200-mL portions of phosphate buffer in saline (0.9 mM $NaH_2PO_4 \cdot H_2O$, 9.6 mM 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 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 contents in each dialysis bag were freeze-dried and stored at −20 °C.

For preparative chromatography, 80 mg of protein from each of the two varieties was dissolved into 250 mL of phosphate buffer, pH 7.8, 0.03 ionic strength, dialyzed in the same buffer overnight, and absorbed on 4 g of a DEAE-cellulose (Bio-Rad cellex D) bed as described earlier (Neucere and Zeringue, 1987). In these experiments at least one-third of the protein applied was eluted in the isocratic fractions (tubes 1–58) which showed sporadic inhibition of *A. flavus* mycelial growth. These are the pooled fractions that were characterized in the current study.

Analytical Procedures. Separation of native cationic proteins by cathodic polyacrylamide gel electrophoresis, PAGE (Bio-Rad), was conducted on vertical slabs at pH 8.5 according to the procedure of Mikola (1965) using a borate–potassium hydroxide buffer system. The stacking gels contained 4% (w/v) acrylamide, and the running gels contained 7.5% (w/v) acrylamide. Electrophoresis was conducted at constant current of 50 mA/slab for approximately 5 h at 10 °C. SDS–PAGE was conducted according to the method of Laemmli (1970). The gels contained 5% (w/v) acrylamide in the stacking medium and 12% (w/v) acrylamide in the running gel. Bio-Rad markers ranging from 14.0 to 92.5 kDa were employed. The electrophoretic conditions were the same as for cathodic PAGE with reversed polarity. Densitometric tracings for all experiments were made with a Cs-930 Shimadzu scanner at wavelength of 600 mm. All gels were stained with 0.125% Coomassie Blue R (Sigma) in methanol–acetic acid–water (4:2:5 v/v/v) and destained with the same solvent system.

Two-dimensional electrophoresis was performed according to the method of O'Farrell (1975) by the Kendrick Laboratory (Madison, WI) as follows: Isoelectric focusing using 1.5% pH 5–7, 1–5% pH 5–8, and 1.0% pH 3.5–10 ampholines (LKB Instruments, Baltimore, MD) was carried out for 9600 V h (700 V for 13 h 45 min). Forty nanograms of an IEF internal standard, vitamin D dependent calcium binding protein, MW 27 000 and p*I* = 5.2, was added to the samples. This standard is indicated by an arrow (M) on the stained 2-D gel. The final tube gel pH gradient extended from pH 4.1 to pH 8.1 as measured by a surface pH electrode (Bio-Rad) and colored acetylated cytochrome p*I* markers (Calbiochem-Behring, La Jolla, CA) run in an adjacent tube. The following proteins (Sigma Chemical Co., St. Louis, MO) were added as molecular weight standards to the agarose which sealed the tube gels to the slab gels: myosin (220 000), phosphorylase A (94 000), catalase (60 000), actin (43 000), and lysozyme (14 000). These standards appear as fine horizontal lines on the silver stained 10% acrylamide slab gels (Oakley et al., 1980). The silver-stained gels were placed in 8% glycerol and transparency dried.

Protein concentrations were determined according to either the BCA method (Pierce) using ovalbumin (Sigma) as standard or the micro-Kjeldahl technique.

RESULTS AND DISCUSSION

One-dimensional electrophoretic profiles and semiquantitation of the native cationic protein bands by densitometry are described in Figure 1. The densitometric tracings showed differences in relative concentration and migration of individual bands as recorded in Table I. The data showed that a large proportion of total proteins was associated with the leading front of electrophoresis (bands 12 in A and B) from both Yellow Creole and Huffman. In

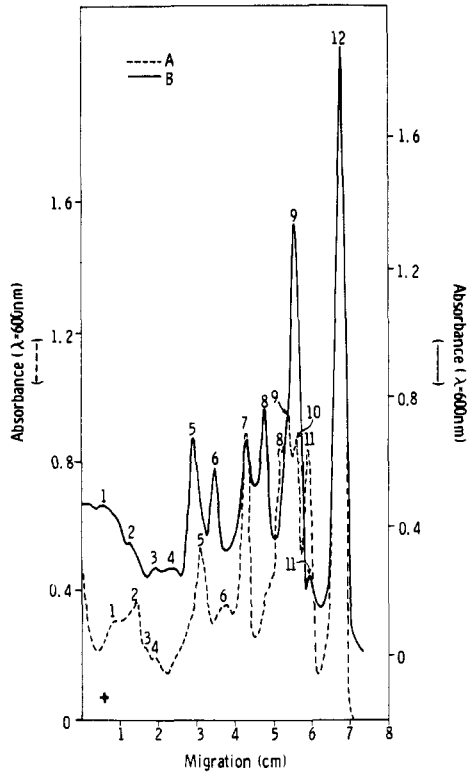

Figure 1. Cathodic PAGE of native cationic proteins from two genotypes of corn [A, Yellow Creole (resistant); B, Huffman (susceptible)]. Densitometric traces of the gels were stained with Coomassie Blue R. Each sample slot was filled with 50 μg of protein. The origin of migration corresponds to the start of the separating gel, and the last peak in each tracing corresponds to the leading edge of electrophoresis. The relative concentration of each peak is described in Table I. The absorbance values (ordinates) for each genotype are staggered to enhance visualization of the tracings.

Table I. Semiquantitative Comparison of Native Cationic Proteins in Yellow Creole (Resistant) (A) and Huffman (Susceptible) (B) Corn from Gel Tracings in Figure 1

peak		migration, cm		% area	
A	B	A	B	A	B
1	1	0.8	0.3	0.6	3.6
2	2	1.5	1.1	7.2	2.2
3	3	1.7	1.7	4.2	0.5
4	4	2.1	2.2	2.6	0.7
5	5	3.1	2.7	11.0	10.6
6	6	3.8	3.2	7.0	6.5
7	7	4.3	4.0	12.0	11.9
8	8	5.2	4.6	8.5	13.0
9	9	5.4	5.3	9.5	19.0
10	10	5.6		8.0	
11	11	6.0	6.0	8.2	4.0
12	12	6.8	6.8	33.0	26.0

particular, the series of bands at CM 5.0–6.5 in Yellow Creole (A) are distinct from those in Huffman (B) in that area of migration. Furthermore, peaks 9 and 10 in Yellow Creole appear as unresolved peak 9 in Huffman. The first seven peaks in the two varieties showed both slight (a) differences in electrophoretic migration and (b) relative concentration variations.

Assessment of molecular weights by SDS–PAGE of the dissociated proteins from the two varieties and relative concentrations of individual bands is shown in Figure 2 and Table II. For both varieties molecular size ranged between 11 and 42 kDa. A major peak of 26.5 kDa in Huffman (peak 5 in Figure 2, trace B) at CM 6.5 was not observed in large quantity in Yellow Creole. In addition, some differences were observed in the two profiles at CM

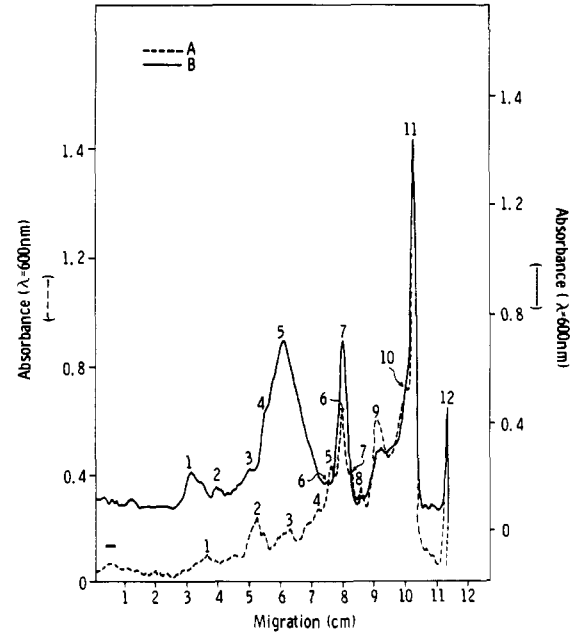

Figure 2. SDS–PAGE of the cationic proteins in the two corn genotypes [A, Yellow Creole (resistant); B, Huffman (susceptible)]. Densitometric scans of gel patterns start at the origin of the separating gel. Molecular weight markers (20 μg) ranging from 14 000 to 92 500 were used to establish a standard curve. The last peak in each tracing corresponds to the leading edge of electropheresis. Each test sample slot was loaded with 50 μg of protein along with Bromophenol Blue as a tracking dye. Relative concentration of each peak and estimated molecular weight are given in Table II. Staggered absorbance values enhance comparison of tracings.

Table II. Semiquantitation and Molecular Weight Determination of Dissociated Cationic Proteins in Yellow Creole (Resistant) (A) and Huffman (Susceptible) (B) Corn from Gel Tracings in Figure 2

peak		% area		molecular weight	
A	B	A	B	A	B
1	1	1.6	3.5	44 000	41 500
2	2	4.9	1.2	30 000	37 000
3	3	2.8	1.5	24 500	32 000
4	4	4.6	4.5	22 000	29 500
5	5	7.2	34.1	20 500	26 500
6	6	8.6	1.2	19 500	21 500
7	7	3.1	11.4	18 500	19 500
8	8	4.4	1.0	17 500	17 800
9	9	14.1	7.6	16 000	16 500
10	10	13.2		14 000	
11	11	12.9	17.5	13 000	13 000
12	12	3.0	2.7	11 500	11 500

8.0–11.5. It appears, for example, that missing peak 10 in trace B is actually unresolved and part of peak 11. These results, therefore, suggest differences in the resolution of dissociated forms of these proteins in the two genotypes.

The distribution of the proteins across two-dimensional gels is shown in Figure 3. The pattern for Yellow Creole (A) is significantly different from that of Huffman (B). There is a group of proteins with isoelectric points between pH 7.0 and 8.0 around 94 kDa (designated "a" in Yellow Creole) that was not observed in the Huffman assay. In addition, a group of spots at pH 4.8–6.2 around 14 kDa (area b) in Yellow Creole were not observed in Huffman. In Huffman, a cluster of proteins (area c) at around 30 kDa and pH 7.0–8.5 did not exist in Yellow Creole. The mapping of protein spots in the two genotypes clearly showed variability either in intensity of staining, in the presence/absence of proteins, or in both of them. Over 50 spots were resolved and detected by visual inspection at

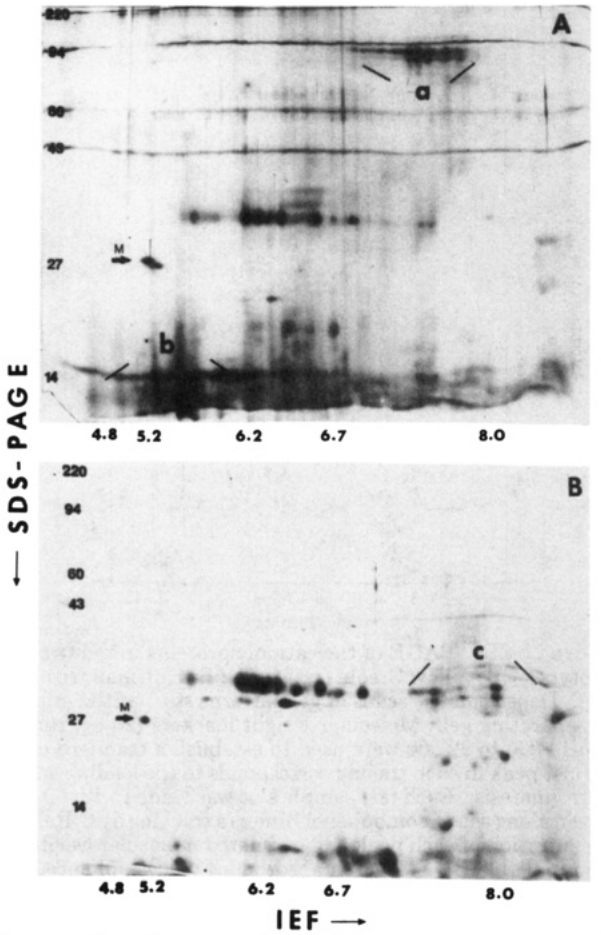

Figure 3. Two-dimensional PAGE of cationic proteins from the two corn genotypes [A, Yellow Creole (resistant); B, Huffman (susceptible)]. Isoelectric focusing (IEF) was used in the first dimension and SDS electrophoresis for the second dimension. Numbers on the left correspond to the molecular weight markers, and M (arrow) denotes the IEF internal standard, MW 27 000. p*I* = 5.2. The lower case letters a–c denote areas of major differences in the profiles of the two genotypes. Lines across the gels correspond to specific molecular weight zones of the markers.

the concentration of protein applied to the isoelectric focusing gels.

In summary, this paper presents a cartography of a group of cationic proteins in kernels of two genotypes of corn that are resistant and susceptible to *A. flavus* contamination. Both one- and two-dimensional gel electrophoretic profiles showed the extent of heterogeneity among the proteins that allow for differentiation of the two genotypes. The results point out that genetic factors responsible for variations in protein amount and presence/absence may be an important basis for recognizing fungal inhibiting proteins that might impart resistance to corn. A comprehensive picture, however, would require protein analyses of a large number of genotypes of corn whose resistance to *A. flavus* infection has been established in laboratory and/or field studies or analysis of corn progeny in which these proteins and aflatoxin resistance are segregating.

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