Surface Proteins of Two Aflatoxin-Producing Isolates of *Aspergillus flavus* and *Aspergillus parasiticus* Mycelia. 2. HPLC Mapping by Gel-Permeation, Ion-Exchange, and Reverse-Phase Chromatography

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Surface mycelial proteins/peptides of *Aspergillus flavus* and *Aspergillus parasiticus* were extracted with phosphate-buffered saline and mapped by several chromatographic procedures. Molecular weights of mycelial proteins ranged from 200 Da to 100 kDa with major differences between species in the distribution of specific protein peaks. Cation exchange chromatography yielded at least 19 peaks for both fungal species, and at least two proteins, following isocratic elution from *A. flavus*, not detected in *A. parasiticus* mycelia were resolved by this procedure. Anion-exchange chromatography resolved 20 and 22 protein peaks in *A. flavus* and *A. parasiticus* extracts, respectively, but sizes and distribution of peaks of both basic and acidic proteins differed. Reverse-phase chromatography showed over 45 peaks in both fungi and revealed a cluster of 10 peaks in *A. parasiticus* that was not observed in *A. flavus*. These elution profiles reveal protein heterogeneity that might have application in the field of fungal taxonomy.

INTRODUCTION

Biochemical means to distinguish isolates and species of *Aspergillus* is extremely important both taxonomically and for practical reasons regarding food/feed safety. Certain morphological and cultural traits are commonly exhibited by aflatoxin producers of both *Aspergillus flavus* and *Aspergillus parasiticus* (Klich and Pitt, 1985; Klich and Pitt, 1988; Murakami and Suzuka, 1970; Parrish et al., 1966). However, other researchers showed that several chemical parameters are not correlated with aflatoxin production using several isolates of the *A. flavus* group (Gupta et al., 1970; Hesseltine et al., 1970; Rambo and Bean, 1974). Hence, the electrophoretic studies referred to in part 1 of this series, the immunochemical profiles reported in part 1, and the literature in general reveal the complexity of establishing chemical/biochemical parameters for correctly identifying isolates of *A. flavus* and *A. parasiticus* in relation to aflatoxin production.

The purpose of this second phase of our study was to establish an overview of chromatographic properties of the surface proteins in *A. flavus* and *A. parasiticus*. We describe HPLC separation and maps of the surface proteins/peptides from two isolates by gel-permeation (GPC), ion-exchange (IEC), and reversed-phase (RPC) chromatography. This information forms a base for determining if similarities and differences in elution profiles can be used to categorize isolates and species of aflatoxin-producing fungi.

MATERIALS AND METHODS

Fungal Isolates. *A. flavus* SRRC (100A) and *A. parasiticus* SRRC (2004), both known to produce aflatoxins, were kindly provided by Dr. M. A. Klich from our laboratory.

Mycelial Growth and Extractive Procedures of Surface Antigens. Spores were maintained on potato dextrose agar as described earlier (Neucere and Zerinque, 1987). Fungal suspensions of 100×10^7 spores/mL were made in 0.001% Triton X-100 for both strains. Mycelia were prepared by inoculating 100 mL of 4% sucrose and 2% yeast extract (Scott Laboratories, Inc.) with 1.0 mL of each spore suspension in 500-mL Erlenmyer flasks. After incubation for 6 days at 28 °C on a rotary shaker (150 rpm), mycelia were collected by filtration through cheese

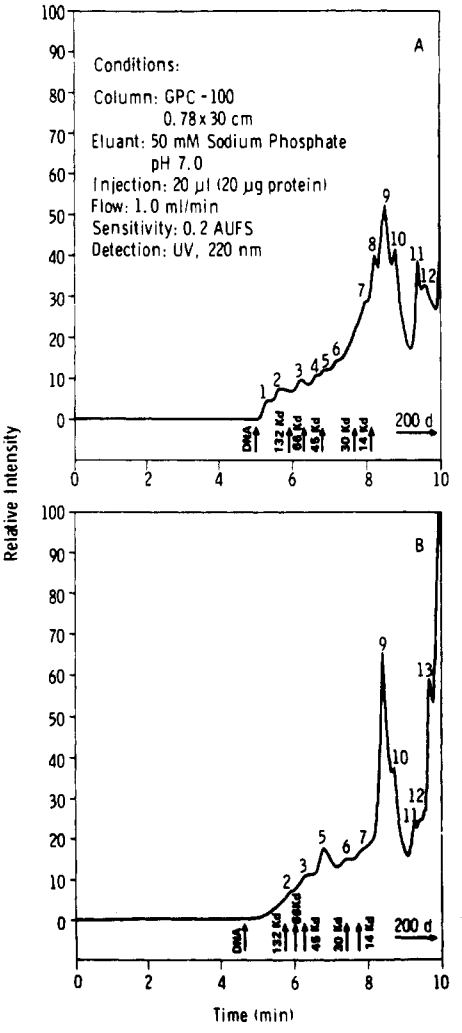

Figure 1. Molecular weight screening of *A. flavus* (A) and *A. parasiticus* (B) mycelial proteins by GPC. Molecular weight markers: 14 000 Da, lysozyme; 30 000 Da, carbonic anhydrase; 45 000 Da, ovalbumin; 66 000 Da, bovine serum albumin (monomer); 132 000 Da, bovine serum albumin (dimer); 300 000, DNA. Conditions are given in part A.

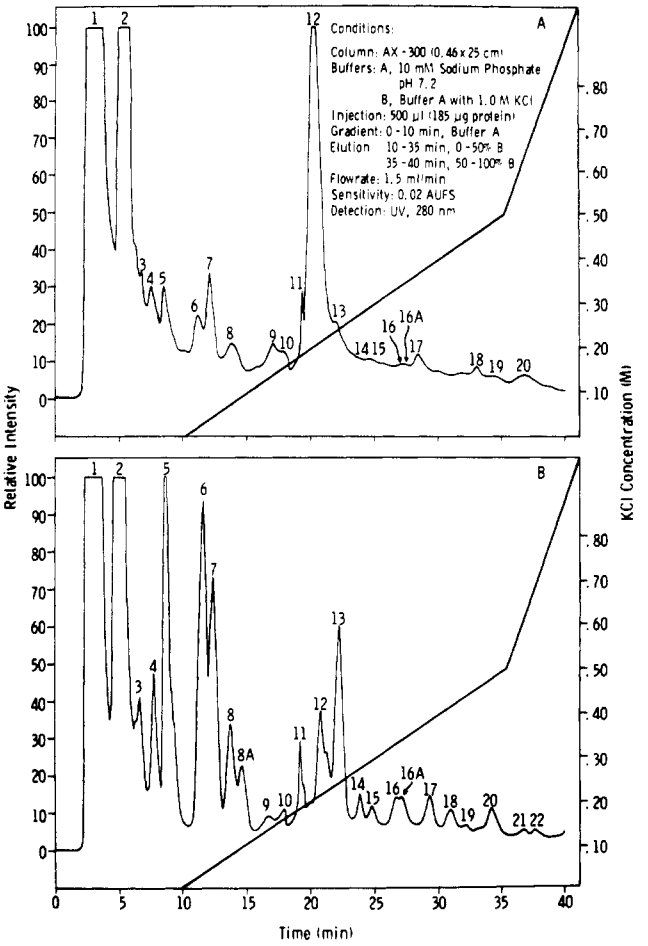

Figure 2. Elution profiles of *A. flavus* (A) and *A. parasiticus* (B) mycelial proteins following anion-exchange chromatography. Conditions of elution are described in part A.

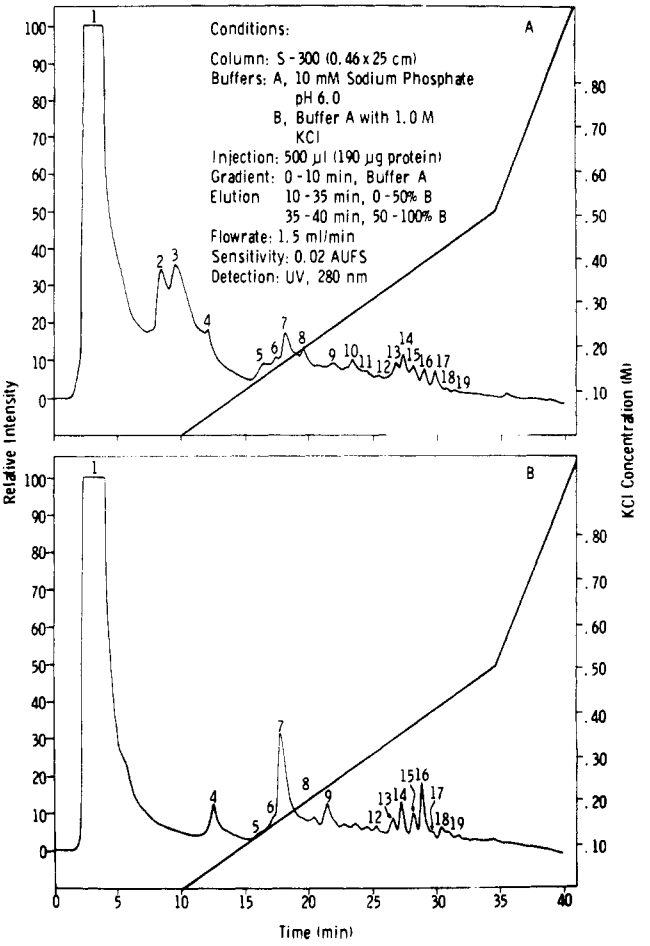

Figure 3. Comparative analyses of mycelial protein extracts of *A. flavus* (A) and *A. parasiticus* (B) eluted from a cation-exchange column. Procedure is described in part A.

cloth and washed several time with cold deionized water. Surface proteins were extracted by stirring 100 mg of intact mycelia in 5 mL of 0.156 M phosphate-buffered saline (PBS), pH 7.2 (Hierholzer and Suggs, 1969) for 3 h at 25 °C. Supernatants were filtered through Whatman no. 2 paper and clarified by centrifugation at 12000*g* for 3 min at 10 °C.

HPLC Conditions. The HPLC System from ISCO (Lincoln, NE) consisted of a V^4 absorbance detector and Model 2350 pump. Data acquisition and analysis, as well as system control, were accomplished by using ISCO's Chemresearch software with an IBM System 2, Model 30, computer (Engel et al., 1989). All of the columns were Synchropak obtained from Synchrom, Inc. (Lafayette, IN). The conditions of analyses are described on each figure.

Chemicals. All chemicals were either reagent or analytical grade unless otherwise specificized.

Protein Estimation. All samples were assayed for protein either by the Lowry et al. (1951) procedure or the BCA (Pierce) method using ovalbumin as standard.

RESULTS AND DISCUSSION

The comparative elution profiles to estimate protein molecular weights in the two species are shown in Figure 1. For both species, the majority of the proteins fall in the range of 30 kDa or less. *A. flavus* showed a preponderance of peaks 1, 2, 3, 4, 7, 8, and 11 over *A. parasiticus*. However, *A. parasiticus* documented larger quantities of peaks 5, 9, and 13 than *A. flavus*. These results show the immense heterogeneity and detectable differences for both low and high molecular weight polypeptides in the two fungi. The importance of size and shape as compared with absolute molecular weight must be emphasized in GPC. In general, small molecules with compact structures appear smaller in solution than others with elongated rodlike structures (Bidlingmeyer and Warren, 1988).

Analyses by anion-exchange chromatography are described in Figure 2. The isocratic fraction containing the cationic proteins at pH 7.2 were eluted by 10 min; five peaks are identified within this region. Peaks numbered 4 and 5 are at higher concentrations in *A. parasiticus* than in *A. flavus*. In general *A. parasiticus* has higher concentrations of anionic proteins than *A. flavus* as evidenced by relative concentrations of peaks 6, 7, 8, 13, 16, 18, and 20 in the two fractions. However, peak 12 in *A. flavus* is the dominant peak within the elution range. In addition, more peaks were discerned in *A. parasiticus* in the range of 0.25 to 0.5 M KCl.

The isocratic fractions containing the anionic proteins at pH 6.0 (Figure 3) were eluted by 10 min. Three peaks were identified in *A. flavus* in this region but only one peak in *A. parasiticus*. Judging from the elution profile, it appears that the majority of the proteins (>75%) were eluted before the onset of the gradient, indicating the high content of anionic species at this pH. However, of all the peaks beyond peak number 4, peaks 7, 9, 14, and 16 were more pronounced in *A. parasiticus* than in *A. flavus*. Other than these observations, the two profiles were very similar.

Separation by RPC, C-18, is shown in Figure 4. Results showed 46 discernible peaks for both species of the fungus. However, the protein/peptide C-18 maps of *A. flavus* and *A. parasiticus* were noticeably different. Of the peaks eluted up to 13 min, two (no. 4 and no. 6) in *A. flavus* were

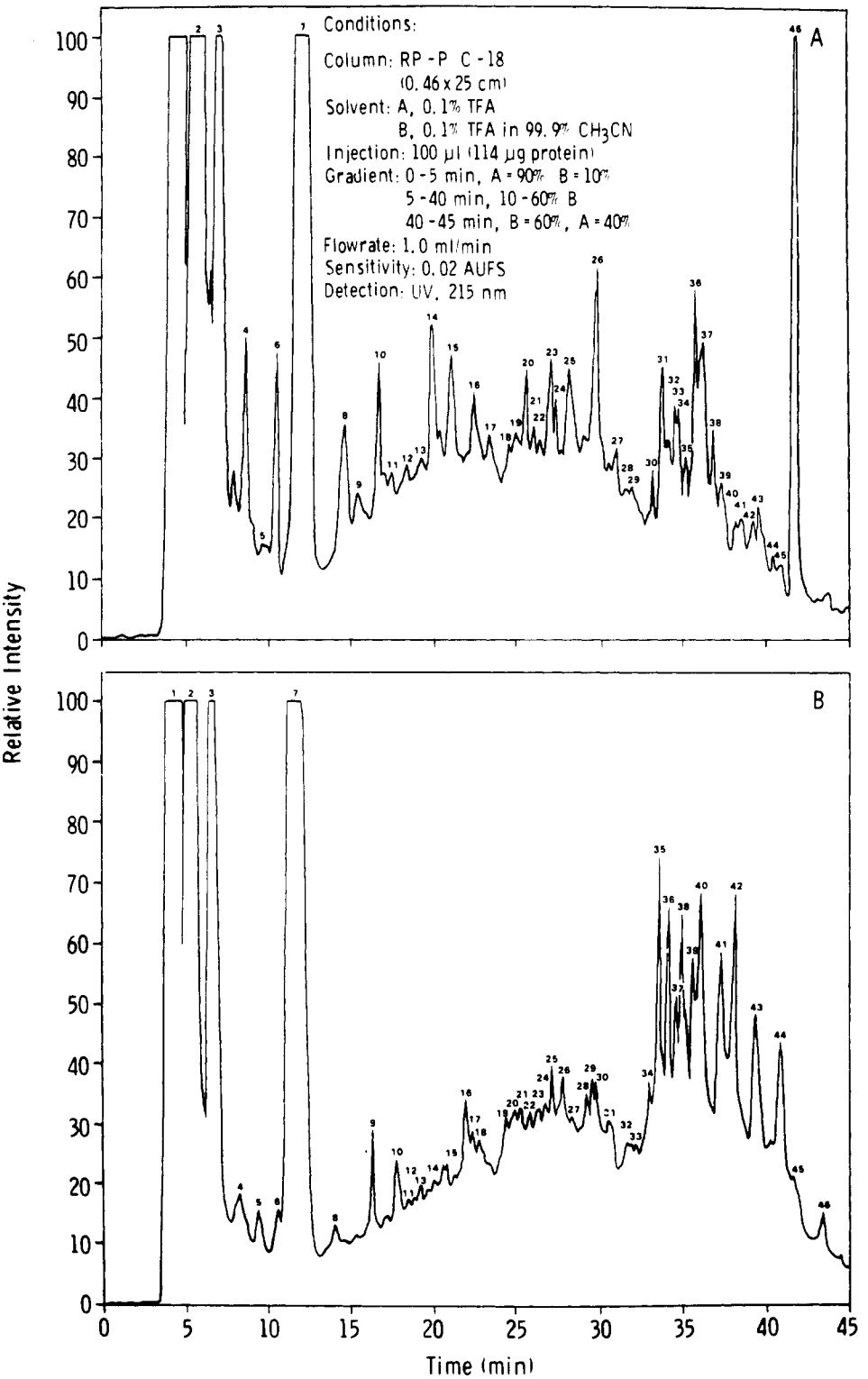

Figure 4. Polypeptide mapping of mycelial proteins from *A. flavus* (A) and *A. parasiticus* (B) employing the reverse-phase mode. Conditions are described in part A.

of higher concentration than in *A. parasiticus*. Furthermore, peaks numbered 8, 10, 14, 26, and 36 were more pronounced in *A. flavus* than in *A. parasiticus*. The most striking difference in the two maps, however, was evident in peaks 26 through 46. For example, peaks 35 through 44 appeared as a cluster in *A. parasiticus* that was not so obvious in *A. flavus*. Finally, peak 46 was a major protein/peptide in *A. flavus* but not in *A. parasiticus*.

In summary the surface proteins/peptides from two species of *Aspergillus* were analyzed by four different modes of HPLC. GPC results indicated that most of the surface antigens had molecular weights less than 30 kDa. Major differences were observed in the weak and strong anionic proteins/peptides in the two fungal species. Some differences were also observed in both the weak and strong cationic proteins/peptides in the two fungi. Striking differences were identified in the reversed-phase mode protein/peptide profile. The overall study showed that the reversed-phase mode of separation provides an elegant "fingerprint" of the surface proteins/peptides in *A. flavus* and *A. parasiticus*. Perhaps this information can be useful as an adjunctive method for categorizing isolates and

species of the fungi and establishing the aflatoxigenic potential of isolates and mutants within both species.

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