27695-7601. Mention of company or trade names is for description purposes only and does not imply endorsement by the North Carolina Agricultural Research Service or by the U.S. Department of Agriculture to the exclusion of others not cited. The support of the North Carolina Tobacco Foundation, Inc., for D.G.S. is gratefully acknowledged.

Registry No. β-HBA, 300-85-6; β-PLA, 156-05-8; succinic acid, 110-15-6.

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Qualitative and Quantitative Changes in the Protein Composition of Peanut (*Arachis hypogaea* L.) Seed following Infestation with *Aspergillus spp.* Differing in Aflatoxin Production

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Peanut (*Arachis hypogaea* L.) seed CV. Early Bunch was inoculated with four different *Aspergillus* lines (NRRL 2999, NRRL 502, NRRL 3357, NRRL 3239) differing in their aflatoxin production capacities. Analyses of the seeds showed that *Aspergillus spp.* infestation caused a decrease in oil, iodine value, soluble carbohydrates, and protein content. Gel filtration studies indicated changes in the seed protein composition while one-dimensional gel electrophoresis revealed that following fungal infestation arachin became more acidic accompanied by the appearance of a basic protein. Two-dimensional gel electrophoresis showed gradual disappearance of a high molecular weight (70 000) polypeptide with an apparent p*I* between 5.8 and 6.4. In addition, several polypeptides with molecular weights between 16 000 and 34 000 also appeared after 9 days of infestation.

Under favorable conditions of temperature and moisture, *Aspergillus spp.* infestation causes rapid changes in seed composition and quality. It has been demonstrated that the seed components such as proteins and carbohydrates not only serve as nutrient source for fungi during their invasion (Krupa and Branstrom, 1974; Zscheile, 1974) but are also involved in aflatoxin biosynthesis (Buchanan and Lewis, 1984; Shih and Marth, 1974). The seed components that are affected by *Aspergillus spp.* infestation include dry matter, protein, oil, fatty acids, carbohydrates, and amino acids (Ward and Diener, 1961; Deshpande and Pancholy, 1979; Cherry et al., 1975; Cherry and Beuchat, 1976). Previous studies have indicated that major storage proteins are converted to numerous low molecular weight components following fungal invasion (Cherry et al., 1976; Cherry et al., 1978). In addition to proteins, enzymes such as esterase, leucine aminopeptidase, catalase, alcohol dehydrogenase, alkaline phosphatase, glucose 6-phosphate dehydrogenase, mannitol dehydrogenase, and malate dehydrogenase are also affected by fungal infestation (Cherry et al. 1978; Buchanan and Lewis, 1984; Cherry et al., 1972). Earlier studies were mainly aimed at determining the overall changes in the protein and enzyme patterns and thus made no special attempt to determine the changes in specific proteins and to identify the seed proteins/polypeptides that may be preferentially utilized by the fungi. Hence, this study was initiated to identify the

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proteins/polypeptides that may be selectively degraded by the invading fungi and also to determine possible differences in host protein utilization by *Aspergillus spp.* differing in aflatoxin production capacities.

MATERIALS AND METHODS

Inoculation. A 20-g portion of hand-shelled seed samples of peanut variety Early Bunch was placed in a Petri dish and surface sterilized by soaking for 5 min in 2.6% sodium hypochlorite (Deshpande and Pancholy, 1979). Soaking was followed by several washings (five to six changes) until the smell of chloride disappeared. The seeds were then transferred to a sterile Petri dish, and then sterile glass-distilled water was added to approximately 20% moisture on a seed weight basis. Three replicates of peanut samples were inoculated with 1 mL of the inoculum: *Aspergillus parasiticus* NRRL 2999 (high aflatoxin producer), *Aspergillus parasiticus* NRRL 502 (no aflatoxin producer), *Aspergillus flavus* NRRL 3357 (high aflatoxin producer), and *Aspergillus flavus* NRRL 3239 (no aflatoxin producer). All fungi inoculants had approximately 5×10^6 spores/mL from 3-week-old cultures grown on Czapek's agar. The control plates received 1 mL of sterile glass-distilled water in the place of *Aspergillus spp.* inoculum. The plates were closed, swirled gently to distribute inoculum, sealed with parafilm, and incubated at 30 °C for 1, 3, 6, 9, 12, 15, and 18 days. After the desired incubation period, seeds were washed thoroughly with water, lyophilized, and ground into a meal. The meals were defatted by extracting with diethyl ether, and the defatted meals were stored at −20 °C until used.

Growth Characteristics. Colonization of peanut samples by the fungus *A. flavus* and *A. parasiticus* was visible after 2 days of incubation and progressed rapidly thereafter. Within 1 week, all inoculated plates were covered with mycelia. Peanut seeds in the uninoculated plates showed no infestation.

Total Nitrogen. Total nitrogen content of the defatted meals was determined by micro-Kjeldahl analysis following the AOAC (1970) method. The percent nitrogen was multiplied by 5.46 to obtain the total protein.

Soluble Protein. The defatted peanut meals (200 mg) were extracted with 50 mM Tris-HCl, pH 8.2, to solublize the seed proteins. The protein extracts were then analyzed for soluble protein by the method of Lowry et al. (1951).

Soluble Carbohydrates and Free Amino Acids. For the free amino acid and soluble carbohydrate determination, 200 mg of defatted meals was extracted with hot methanol–chloroform–H_2O (60:25:15, v/v/v) and centrifunged at 20000*g* for 20 min. The supernatants were used for estimating the content of carbohydrates by the anthrone–H_2SO_4 method (Yemm and Willis, 1955) and the free amino acids by the ninhydrin method (Yemm and Cocking, 1955).

Gel Filtration. Peanut meals (3 g) were extracted with 10 mL of 2 M NaCl, 10 mM Tris-HCl, pH 8.2, and 2 mM phenylmethanesulfonyl fluoride and then centrifuged at 20000*g* for 20 min. The resulting supernatant was loaded on a Sephacryl S-300 column (2.5 × 135 cm) and eluted with a buffer containing 0.5 M NaCl, 10 mM Tris-HCl, pH 8.2, and 0.02% NaN_3, and the effluent was collected in 5-mL fractions. Protein content of these fractions was monitored by measuring their absorption at 280 nm.

One-Dimensional Gel Electrophoresis. Protein from the defatted meal was extracted with 0.5 M NaCl and 10 mM Tris-HCl buffer, pH 8.2, and then subjected to electrophoresis under nondenaturing conditions in 7.5% (w/v) polyacrylamide gels as described by Davis (1964). About 50 μg of protein was loaded on each gel and then electrophoresed toward the anode. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed after dissociating the samples by boiling for 3 min in a buffer containing 2% (w/v) SDS, 1.5% (w/v) dithiothreiotol, and 1.2% (w/v) Tris. The dissociated proteins (100 μg) were electrophoresed in 10% acrylamide gels (Laemmli, 1970) containing 0.1% SDS. Following electrophoresis, the proteins were stained with 0.1% Coomassie Blue R-250 in 7% acetic acid and 10% ethanol and then destained in 7% acetic acid containing 10% ethanol. The destained gels were scanned in a Beckman Model 25 spectrophotometer equipped with a gel scanner, using a 0.05-mm slit.

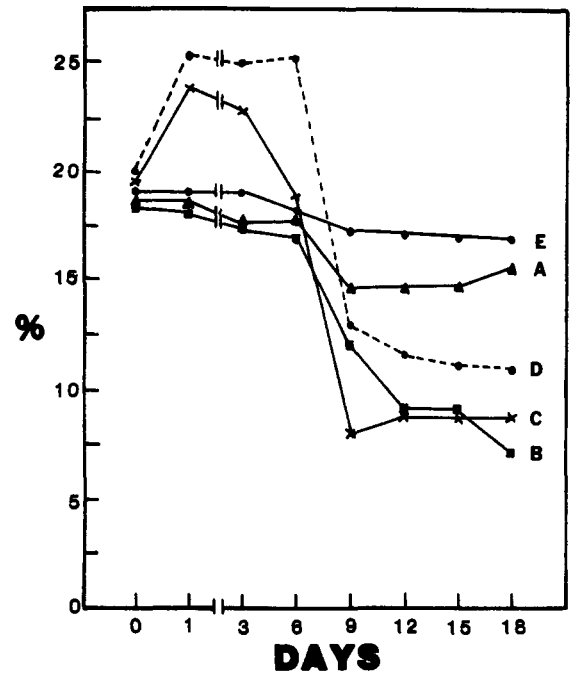

Figure 1. Changes in the soluble protein content of peanut seed following infestation with different *Aspergillus spp.*: A = NRRL 2999; B = NRRL 502; C = NRRL 3357; D = 3239; E = control.

Two-Dimensional Gel Electrophoresis. Changes in the protein composition of the seed were monitored by two-dimensional gel electrophoresis (2-D PAGE) as described by Basha (1979). For this purpose the defatted meal was extracted with a solution containing 9.3 M urea, 5 mM K_2CO_3, 3% (v/v) 2-mercaptoethanol, and 2% (v/v) nonionic detergent NP-40. The extracts were then subjected to isoelectric focusing in 4% (w/v) acrylamide gels containing 9.3 M urea and a 2% (v/v) ampholine mixture (pH 3.5–10, 5–7, 9–11 ampholines, 50:34:16). Following IEF, the gels were equilibrated with a solution containing 0.1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and 65 mM Tris-HCl, pH 6.9, and then subjected to SDS slab gel electrophoresis in 10% (w/v) acrylamide gels. After electrophoresis the proteins were fixed in 7% acetic acid and 40% ethanol and then stained with Coomassie Blue R-250.

RESULTS AND DISCUSSION

Oil, Total Nitrogen, and Iodine Values. During the 18-day incubation period, the uninoculated peanut seeds did not show any significant changes in oil content, total N, and iodine values. However, the inoculated seeds showed changes in oil, total N, and iodine values that were similar (data not shown) to the previous findings (Ward and Diener, 1961; Deshpande and Pancholy, 1979; Cherry et al., 1975; Cherry and Beuchat, 1970).

Soluble Proteins. The soluble protein content of the peanut seeds decreased during the 18 days of fungal in-

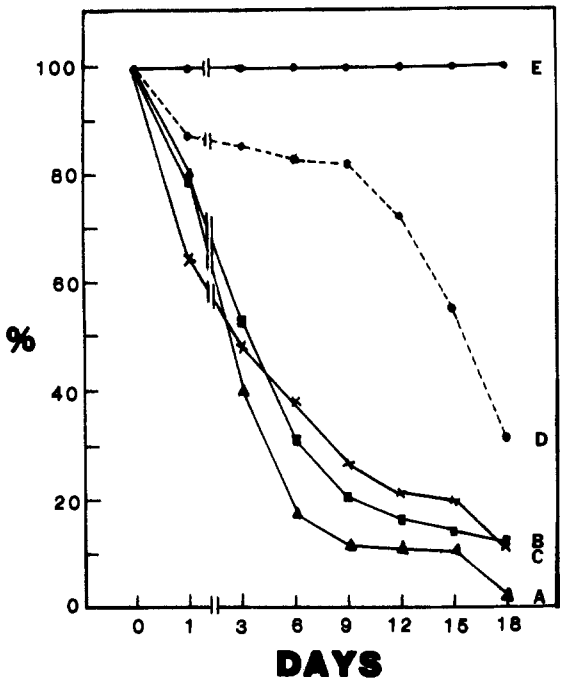

Figure 2. Changes in the soluble carbohydrate content of the peanut seed following infestation with various *Aspergillus spp.*: A = NRRL 2999; B = NRRL 502; C = NRRL 3357; D = NRRL 3239; E = control.

festation (Figure 1). In *A. parasiticus* (NRRL 2999, NRRL 502) the protein content decreased gradually during the first 6 days of infestation followed by a rapid decline afterward. Interestingly, in the *A. flavus* lines (NRRL 3357, NRRL 3239) the protein content increased (4–5%) during the initial 3–6 days of inoculation prior to decreasing to 18 days. This increase may be because of the higher solubility of the seed proteins resulting due to their breakdown into lower molecular weight polypeptides by the fungal proteases. In the control only a slight decrease (1.75%) in the protein content was observed over the 18-day period.

Soluble Carbohydrates and Free Amino Acids. The soluble carbohydrate content of the seeds dramatically decreased with the increasing period of incubation (Figure 2). Rapid decline (about 80%) occurred between 0 and 6 days after inoculation, and by day 18 more than 90% of the seed carbohydrates disappeared. This would indicate that the fungi utilized the seed carbohydrates rapidly during the initial stages of infestation. Carbohydrate utilization patterns were similar in all the fungi except for NRRL 3239 (no aflatoxin producer), which showed relatively slower carbohydrate utilization. Unlike the carbohydrates, the free amino acid content of the seed increased during the fungal infestation (not shown) as a result of seed protein breakdown by the fungal proteases.

Gel Filtration. Fractionation of seed proteins by gel filtration indicated that *Aspergillus spp.* infestation did not greatly alter the seed protein composition (Figure 3) during the initial stages of infestation. Thus, no major qualitative and quantitative differences were observed in seed protein composition during the early stages of infestation. However, the amount of protein in the salt volume increased gradually during the 18 days of infestation (Figure 3CD), while the control showed (not shown) no major changes in protein composition during this period. Thus, the ratio of arachin (peak II) and salt volume peak (V) was 3.7 on day 0 of incubation and 1.4 on day 18, indicating increasing amounts of low molecular weight

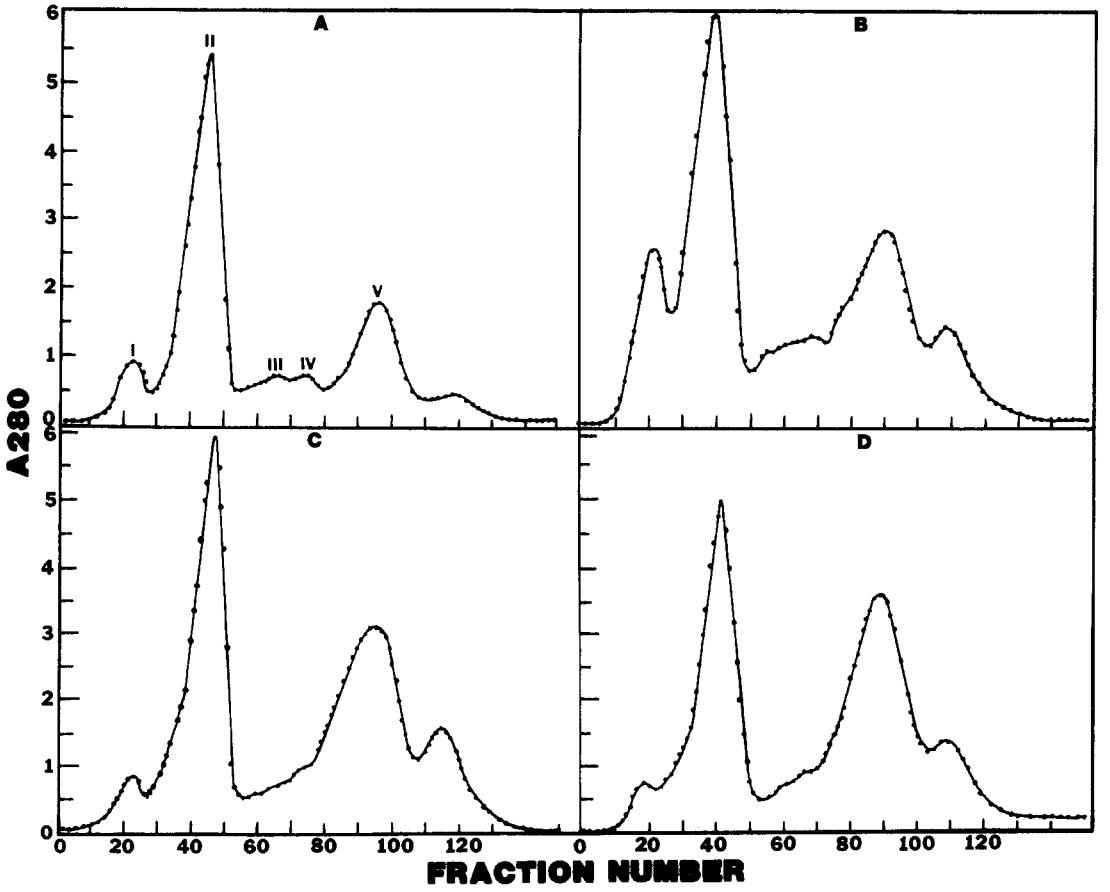

Figure 3. Gel filtration profiles of peanut seed infested with the high-aflatoxin-producing *A. parasiticus*, NRRL 2999: A = 0 day; B = 3 days; C = 6 days; D = 15 days.

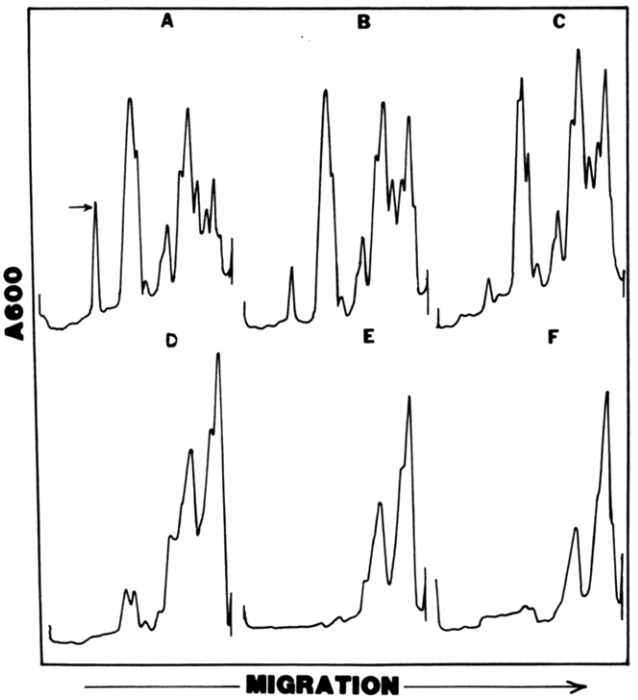

Figure 4. One-dimensional SDS gel electrophoretic profiles of peanut seed infested with high-aflatoxin-producing (NRRL 2999) *A. parasiticus*: A = 0 day; B = 1 day; C = 3 days; D = 6 days; E = 12 days; F = 18 days. About 100 μg of protein was loaded on each gel.

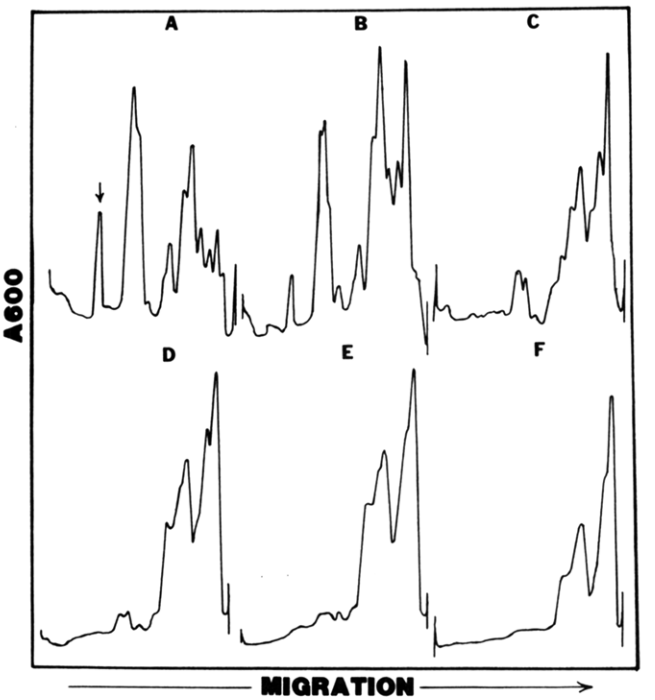

Figure 5. SDS gel electrophoretic patterns of peanut seed inoculated with no-aflatoxin-producing (NRRL 502) line of *A. parasiticus*: A = 0 day; B = 1 day; C = 3 days; D = 6 days; E = 12 days; F = 18 days. About 100 μg of protein was loaded on each gel.

components during infestation. This increase in the low molecular weight proteins may be due to the breakdown of high molecular weight seed reserve proteins by the fungal proteolytic enzymes. Similar gel filtration profiles were observed (not shown) with the other fungal lines.

One-Dimensional Gel Electrophoresis. Following electrophoresis under nondenaturing conditions, peanut proteins resolved into one major arachin band and several minor non-arachin bands. Infestation by the high-aflatoxin-producing line (NRRL 2999) of *A. parasiticus* appeared to have caused no significant changes in the seed protein composition between 0 and 3 days after inoculation (not shown). However, on day 6, the relative mobility of the arachin band had increased from 0.18 (control) to 0.20 accompanied by the appearance of a slightly basic protein before the arachin band. This component may be a breakdown (due to the action of fungal proteases) product of the arachin, loss of which might have made the arachin molecule more acidic. Unlike NRRL 2999, in NRRL 502 infested seeds, changes in protein composition were readily evident on day 3 of infestation, indicating that the no-aflatoxin-producing line utilized the seed proteins more rapidly than the high-aflatoxin-producing line. Similar protein utilization patterns were observed (not shown) with the high-aflatoxin-(NRRL 3357) and no-aflatoxin- (NRRL 3239) producing lines of *A. flavus*.

SDS gel elctrophoresis indicated (Figure 4) no significant changes in the high molecular weight major components during the initial periods of NRRL 2999 infestation. However, a high molecular weight (70 000; shown with an arrow) polypeptide gradually decreased during this period accompanied by increasing amounts of low molecular weight (between 13 000 and 18 000) components. There was a major decrease in the high molecular weight (70 000 and 45 000) polypeptide components from day 6 onward (Figure 4D–F). Unlike the high-aflatoxin-producing line, the no-aflatoxin-producing line NRRL 502 showed (Figure 5) rapid disappearance of the high molecular weight components by day 3. This is consistent with the results observed following electrophoresis under nondenaturing conditions. Similar results were obtained (not shown) with both the *A. flavus* lines.

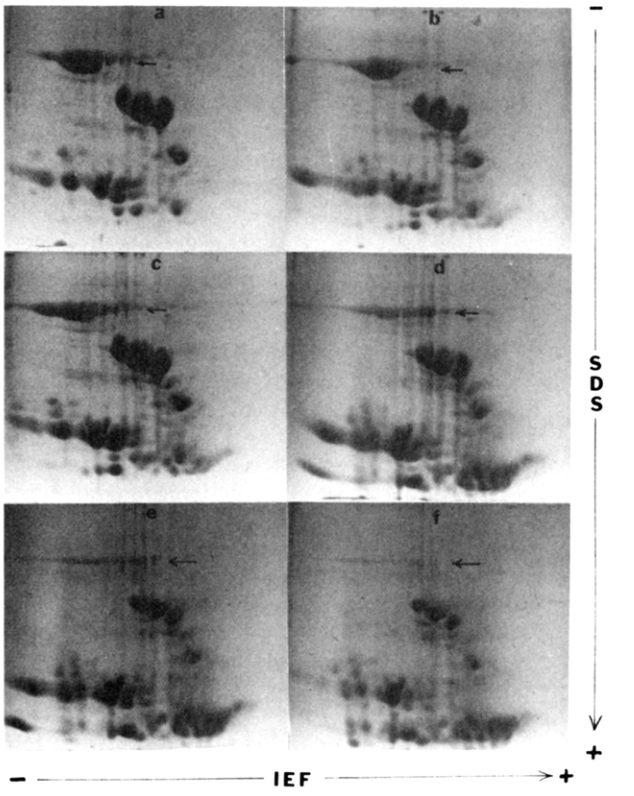

Figure 6. Two-dimensional polyacrylamide gel elctrophoretic profiles of peanut seed infested with *A. parasiticus*, NRRL 2999: A = 0 day; B = 1 day; C = 3 days; D = 6 days; E = 12 days; F = 18 days. About 200 μg of protein was applied on each gel. Arrow indicates the M_r 70 000 polypeptide.

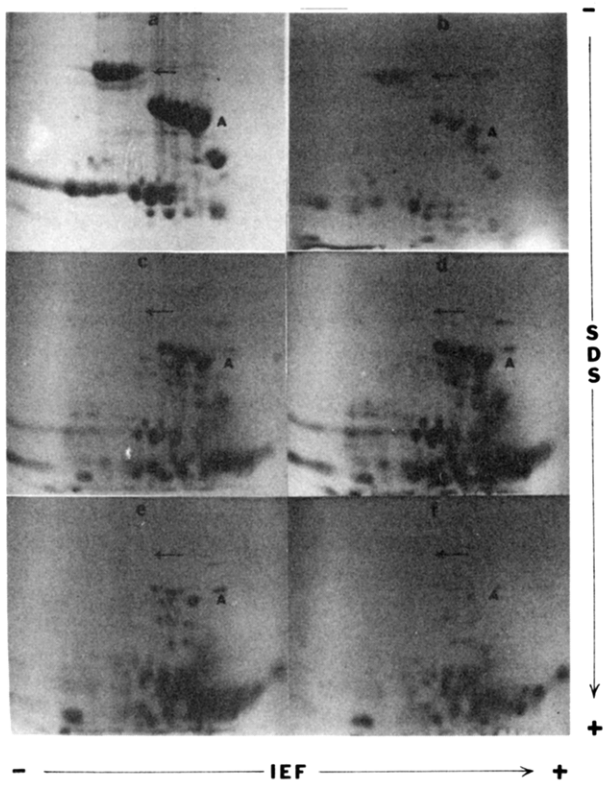

Figure 7. Two-dimensional polyacrylamide gel electrophoretic patterns of peanut seen infested with *A. parasiticus*, NRRL 502: A = 0 day; B = 1 day; C = 3 days; D = 6 days; E = 12 days; F = 18 days. About 200 μg of protein was loaded on each gel. Arrow indicates the M_r 70 000 polypeptide, and A, the M_r 43 000 polypeptide.

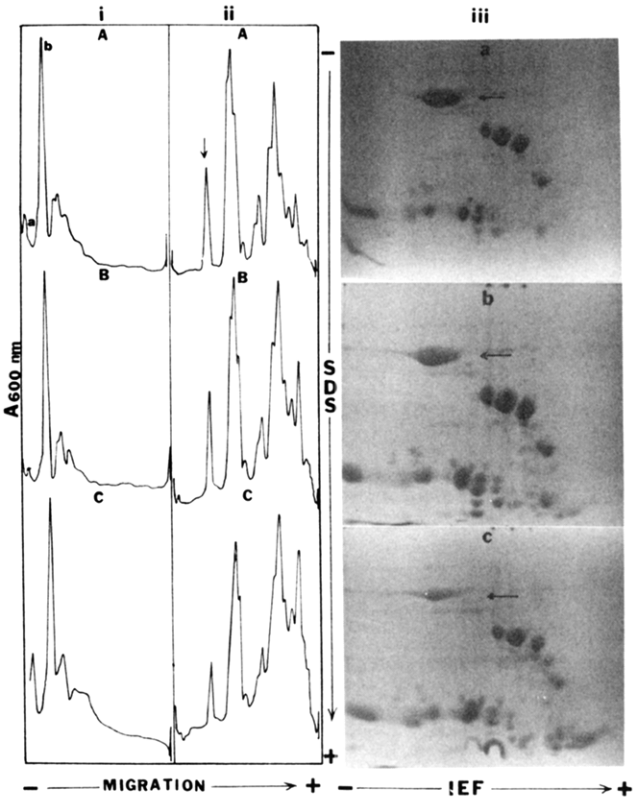

Figure 8. One- and two-dimensional gel electorphoretic profiles of uninoculated peanut seeds: (i = nondenaturing gels) A = 0 day, B = 9 days, C = 18 days; (ii = SDS gels) A = 0 day, B = 9 days, C = 18 days; (iii = two-dimensional acrylamide gels) A = 0 day, B = 9 days, C = 18 days. About 60, 100, and 200 μg of protein, respectively, was loaded on each gel and electrophoresed toward the anode.

Two-Dimensional Gel Electrophoresis (2-D PAGE). Because of multimeric nature of the arachin molecule, the observed changes in the electrophoretic mobility of the native molecule were further examined at the polypeptide level by 2-D PAGE. Two-dimensional gel electrophoresis, which resolves proteins on the basis of their isoelectric point and molecular weight, separated peanut proteins into numerous polypeptides (Figure 6). However, it should be noted that since arachin constitutes more than 70% of the seed protein, the majority of the polypeptide spots visible on the 2-D gel are of arachin (Basha and Pancholy, 1981). As seen in Figure 6 the polypeptide pattern remained relatively unchanged between 0 and 3 days of infestation. However, from day 6 onward a polypeptide (shown with an arrow) with a molecular weight around 70 000 and p*I* between 6.2 and 7.0 rapidly disappeared and was almost absent by 15 days of infestation. In addition, several low molecular weight (between 13 000 and 18 000) polypeptide spots gradually appeared from 6th day of incubation. These polypeptide spots may represent the breakdown products of the high molecular weight polypeptides. Unlike the high-aflatoxin-producing *A. parasiticus* (NRRL 2999), the polypeptide breakdown patterns were more dramatic with the no-aflatoxin-producing *A. parasiticus* (NRRL 502) (Figure 7). As seen in Figure 7 by day 3, the high molecular weight (70 000) polypeptide (shown with an arrow) had disappeared. In addition, a group of arachin polypeptides (Basha and Pancholy, 1981) with molecular weight around 43 000 and p*I* between 5.5 and 6.2 (shown with an A) gradually decreased from day 3 onward and completely disappeared by day 18. However, it should be noted that in the case of *A. parasiticus* (NRRL 2999) these polypeptides were visible even on day 18 of incubation (Figure 6F). Similar results were found (not shown) with both the high-aflatoxin- (NRRL 3357) and no-aflatoxin-producing (NRRL 3239) lines of *A. flavus*.

Thus, the elctrophoresis data indicated that the 70 000 molecular weight polypeptide was more readily utilized by both fungi from the beginning of the infestation. In addition, in both the *A. flavus* and *A. parasiticus*, the no-aflatoxin-producing lines utilized the host proteins more rapidly than the high-aflatoxin-producing lines, indicating that the proteolytic enzymes of the no-aflatoxin-producing lines were more potent than the high-aflatoxin-producing lines, which may be partially responsible for the high aflatoxin production.

Unlike the infested seeds the uninoculated control seeds showed no significant changes in the seed protein composition during the 18-day study period. Thus, as seen in Figure 8, the 1-D and 2-D gel elctrophoreses showed no significant qualitative changes in the protein and polypeptide composition of the uninfested seeds. However, there was a slight decrease in the amount of the 70 000 molecular weight polypeptide after 15 days of infestation. This decrease may be due to the action of endogenous peptidases and/or proteolytic enzymes of the peanut seed that may have been activated or de novo synthesized (Basha and Cherry, 1978) during the longer incubation periods. Additional studies are in progress in order to determine the relationship between drought and temperature stress and aflatoxin production and also to identify biochemical markers for aflatoxin contamination.

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Studies of Lipid–Protein Interaction in Stored Raw Peanuts and Peanut Flours

Allen J. St. Angelo* and Elena E. Graves

Peanuts readily undergo lipid oxidation because of their high polyunsaturated fatty acid content in both their triglycerides and polar lipid components. Defatted peanut flour contains residual lipids, which because of their various reactive groups can affect the ultimate quality of the flour destined for human consumption. The role of these lipids was investigated as to their involvement in protein–lipid interaction. Polar and nonpolar bound lipids were extracted from proteins with an acidic solvent system comprised of chloroform, methanol, and hydrochloric acid. The neutral fraction contained sterols, triglycerides, and esterified and free fatty acids. The polar fraction contained phospho- and glycolipids. The electrophoretic mobilities of the proteins that contained the bound lipids were changed after removal of the lipids by solvent extractions. Results of this study could be useful to understanding an important factor that affects the quality and stability of peanuts, products, and flours stored for long periods before their utilization.

INTRODUCTION

Lipid oxidation is known to be a major problem in the storage of fresh and processed foods. The oxidation process can adversely affect not only flavor, odor, and color qualities but also nutritive value. Lipid peroxides can cause damage to proteins, enzymes, and amino acids. The autoxidized products or precursors can possibly serve as toxic, mutagenic, or carcinogenic agents. These deleterious effects of autoxidation, as well as some that are beneficial, were previously described (Simic and Karel, 1980).

Peanuts readily undergo lipid oxidation because of their high polyunsaturated fatty acid content in both their triglycerides and polar lipid components. Lipoxygenase can oxidize lipids in raw peanuts, but its activity is lost during roasting (St. Angelo et al., 1979). Lipoxygenase, prepared from raw peanuts, was shown to catalyze the oxidation of linoleic acid and its methyl ester to form C-9 and C-13 hydroperoxides (St. Angleo et al., 1980a), which ultimately can degrade into secondary products that cause off-flavors (St. Angelo et al., 1980b). In roasted peanuts, lipids are oxidized by nonenzymic sources (St. Angelo et al., 1979). Hexane-defatted peanut flour was found to contain up to 2% polar lipids, which because of their reactive groups can also affect the ultimate quality of the flour destined for human consumption (St. Angelo and Ory, 1975).

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Lipid–protein interaction was examined in hexane-extracted meals and in the protein extracted from raw and roasted whole peanuts and peanut butter (St. Angelo and Ory, 1975a,b). Results on polyacrylamide gel electrophoresis (PAGE), with the use of a dual staining system, Amido Black for protein and Oil Red O or Rhodamine 6B for lipid, showed that at least three major protein components were associated with lipid. Upon storage, at least one of the lipid bands became noticeably denser and broader than the corresponding band in the fresh control samples. Extraction of the fresh and stored protein samples with hexane or chloroform/methanol (2/1) failed to remove any of the lipids that were associated with the three protein bands. Also, in each of these experiments, the electrophoretic protein banding patterns of extracts from the two solvent systems were identical. As part of our investigation on the effects of peroxidized lipids on food quality, and to better understand the nature of components responsible for adversely affecting food quality, we report in this paper on the interaction between lipids and proteins and a solvent system to remove bound lipids.

MATERIALS AND METHODS

Lipid standards were purchased from Supelco, Inc. (Bellefonte, PA). Catalase (C-10), insulin (I-550), ribonuclease-S-peptide (R-6125), and soybean lipoxygenase (L-7127) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA), albumin fraction V, and egg albumin were purchased from Nutritional Biochemicals (Cleveland, OH). Oil Red O (ORO) was purchased from Matheson, Coleman, and Bell (Nor-