Effects of Oilseed Storage Proteins on Aflatoxin Production by *Aspergillus flavus*

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ABSTRACT: Potential involvement of seed storage proteins in susceptibility to aflatoxin contamination was assessed with *in vitro* tests. Initially, two oilseed storage proteins [cottonseed storage protein (CSP) and zein] were compared with bovine serum albumin (BSA) and collagen. Supplementation of a complete defined medium with either oilseed storage protein resulted in significantly more aflatoxin production by *Aspergillus flavus* than supplementation with either BSA or collagen. Little or no aflatoxin was produced when either BSA, CSP, or zein was employed (at 0.5%) as both the sole carbon and the sole nitrogen source. Media with collagen (0.5%) as the sole nitrogen and carbon source supported aflatoxin production similar to the complete defined medium. Although lower than levels observed with defined medium, aflatoxin production increased with both increasing CSP concentration (0 to 2.0%) and increasing zein concentration (0 to 6.0%) when these proteins served as both the sole carbon and sole nitrogen source. Denaturing polyacrylamide gel electrophoresis and protease activity assays indicated that fungal acquisition of protein carbon was probably *via* hydrolysis mediated by the 35 kD metalloprotease of *A. flavus*. Media lacking nitrogen but containing sucrose (5.0%) and supplemented with either zein (1.7%) or CSP (2.0%) supported three- to eightfold more aflatoxin production than the complete defined medium. The results suggest seed storage proteins, when present with an accessible carbon source, may predispose oilseed crops to support production of high levels of aflatoxins by *A. flavus* during seed infection. *JAOCS 75,* 1085–1089 (1998).

KEY WORDS: *A. flavus,* aflatoxin, corn, cottonseed, cottonseed storage protein, metalloprotease, zein.

The toxigenic fungus *Aspergillus flavus* is a widely distributed saprophyte that, under specific environmental conditions, is capable of opportunistic pathogenesis in plants. As a result, the value of the oilseed crops corn and cottonseed is diminished due to fungal production of the potent carcinogen aflatoxin (1). Although storage proteins are prominent components of both crops, the contribution of these proteins to crop vulnerability to aflatoxin contamination has not been addressed.

Zein, the prolamin (ethanol-soluble) storage protein of corn, is localized in the endosperm, whereas cottonseed storage protein (CSP), a globulin (salt-soluble), is localized in the developing cotyledons. The dry weight composition of protein is 11 to 12% in corn and 39% in cottonseed (2). Zein comprises half of all seed protein in corn and CSP is the major protein in cottonseed (2). In addition, CSP contains 18% nitrogen (3). These seed protein reserves are potential carbon and nitrogen resources for the fungus. Fungal invasion of oilseed crops may produce localized, elevated levels of seed storage proteins (protein bodies) as potential fungal substrates. Proteases capable of degrading a wide array of proteins are widely distributed in *Aspergillus* section *Flavi* (4). The primary enzyme responsible for this protease activity from *A. flavus* has been purified and characterized (5). However, its role in fungal utilization of potential seed protein substrates and in the process of aflatoxin contamination is unknown.

In order to better understand potential contributions of storage proteins in susceptibility to contamination, effects of zein, CSP, and other proteins on aflatoxin production, fungal protease production, and other culture parameters were examined. A preliminary report has been given (6).

EXPERIMENTAL PROCEDURES

Biological materials. Aspergillus flavus AF13 was isolated from soil collected in southern Arizona and maintained on a 5% V-8 vegetable juice (Campbell Soup Co., Camden, NJ) agar at 30°C (7). Culture medium was seeded (200 µL/70 mL) with a conidial suspension containing 10^7 to 10^8 spores per mL. Collagen (Type I), bovine serum albumin (BSA), and zein were purchased from the Sigma Chemical Company (St. Louis, MO). CSP was obtained from defatted cottonseed flour (gift of E.J. Conkerton, Southern Regional Research Center) according to the method of Marshall and Conkerton (8). The protein preparation (in 10% NaCl) was exhaustively dialyzed against deionized water and lyophilized to dryness.

Fungal incubations. The defined fungal medium (9) contained sucrose (50 g/L) as the sole carbon source and sodium nitrate (3 g/L) as the sole nitrogen source (10). Different treatments contained varying amounts of the four test proteins: CSP, zein, BSA, and collagen. In some cases, the fungal medium lacked either sucrose or sodium nitrate or both, but did contain the other components of the defined medium. Fungal cultures were grown by shaking (200 rpm) for 5 d at 30°C (dark) except where noted.

Aflatoxin analysis. Following incubation, cultures were stopped and aflatoxins solubilized with 50% (vol/vol) ace-

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TABLE 1
Effect of Supplementary Protein Substrates on Aflatoxin and Biomass Production in Complete Defined Medium (AM), AM Lacking Sucrose (–C), and AM Lacking Both Sucrose and $NaNO_3$ (–C/N)

	AM		–C		–C/N	
Treatment[a]	AFB_1[b]	Biomass[c]	AFB_1	Biomass	AFB_1	Biomass
Control	160 b	0.55 a	0 c	0 d	0 c	0 d
Bovine serum albumin	224 b	1.47 a	0 c	0.21 b	1.4 c	0.23 b
Collagen	363 b	0.82 a	141 a	0.13 c	314 a	0.16 c
Cottonseed storage protein	1487 a	1.66 a	1.6 c	0.25 a	1.5 c	0.24 b
Zein	948 a	1.06 a	19.9 b	0.26 a	55 b	0.30 a

[a]All protein supplements present at 5 g/L.
[b]Mean aflatoxin (AFB_1) is expressed in µg per flask ($n = 3$). Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's least significant difference (LSD).
[c]Mean biomass (dry weight of mycelium) is expressed in grams per flask ($n = 3$).

tone. Four microliters of the medium-acetone solution was spotted on silica gel G plates which were developed in diethyl ether/methanol/water (96:3:1) mobile phase. Aflatoxin B_1 was quantified directly on thin-layer plates by fluorescence densitometry (11). Mycelial mats were separated by filtration (Whatman No. 1 paper) *in vacuo,* dried at 50°C for 2 d, and weighed. Data for the zein dose-response study was log transformed prior to statistical analysis in order to improve homogeneity among variances.

Elastase assays. Culture filtrates were analyzed for protease activity by means of a radial diffusion assay which employed elastin as the protein substrate (4). Culture filtrates were first treated with a reversed-phase C_{18} cartridge (Sep-Pak; Millipore, Milford, MA) to remove aflatoxin. The C_{18} cartridge was charged with 3 mL of methanol, followed by a wash with 5 mL of deionized water. Aflatoxin quantitatively bound to the cartridge matrix, and the effluent was free of aflatoxin (<1 ng/mL). The effluent was tested directly for protease activity (3-mL sample aliquots).

Electrophoresis. Denaturing polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli (12), except 0.05 M dithiothreitol was substituted for 2-mercaptoethanol in the sample buffer. Culture filtrates (detoxified as above) were used without concentration and were diluted 1:1 (vol/vol) with sample buffer (SDS) prior to the heat treatment. The protein bands were visualized using a silver stain procedure (13).

RESULTS

Addition of relatively low levels of supplementary proteins to the defined medium (9) resulted in some substantial changes in culture parameters. Five grams per liter of either CSP, zein, collagen, or BSA stimulated biomass (dry weight) production. Although there were no differences in biomass production between individual proteins in supplemented (AM) cultures, protein-supplemented cultures in general produced more biomass than defined medium controls. In media lacking a carbohydrate carbon source (–C and –C/N), there were significant differences in biomass production between cultures with different proteins (Table 1). Cultures containing CSP or zein showed a six- to tenfold stimulation of aflatoxin over defined medium without protein supplementation (Table 1). Cultures with only BSA or CSP as a carbon/nitrogen source (no sucrose or $NaNO_3$) produced little or no aflatoxin. Cultures containing collagen as the sole C/N source produced aflatoxin levels similar to the complete defined medium (Table 1). Preliminary experiments also showed that aflatoxin production (40–470 µg/g dry weight) increased with starch concentration (1–20 g/L) in defined medium without sucrose.

In order to further investigate effects of seed storage proteins on aflatoxin production, dose-response experiments were carried out with CSP and zein. Media supplemented with zein (1–15 g/L) supported four- to sixfold more aflatoxin production than defined medium alone. Cultures with either zein or CSP as both sole carbon and sole nitrogen source produced much lower aflatoxin levels than defined medium controls. However, for both proteins, aflatoxin production was directly correlated with initial protein concentration of cultures (Fig. 1, Table 2).

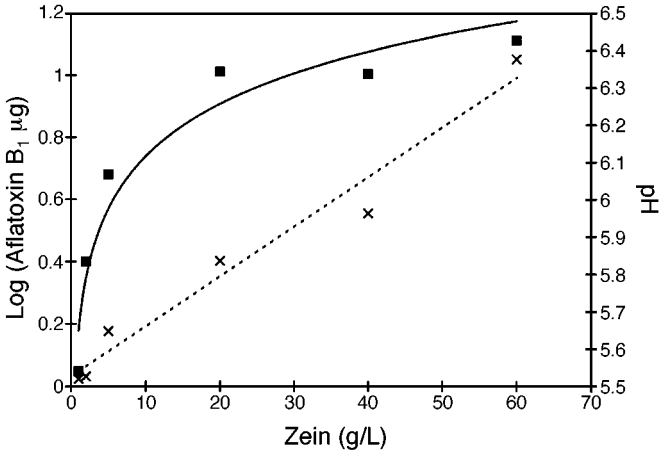

FIG. 1. Effect of zein concentration on aflatoxin production (×) and final culture pH (■). All incubations used complete defined medium without either sucrose or $NaNO_3$. Mean aflatoxin expressed as log µg per flask ($n = 3$; $r^2 = 0.97$, $P < 0.05$).

TABLE 2
Effect of CSP Concentration on Aflatoxin Production, Biomass Production, and Culture pH in a Complete Defined Medium Lacking $NaNO_3$ (–N) or Lacking Both Sucrose and $NaNO_3$ (–C/N)

	–N			–C/N		
Treatment[a]	AFB_1[b]	Biomass[c]	pH[d]	AFB_1	Biomass	pH
CSP, 1 g/L	218 c	0.264 e	3.19 c	0.101 c	0.101 d	5.57 d
CSP, 2 g/L	173 c	0.50 d	3.09 c	0.078 c	0.149 c	5.94 c
CSP, 5 g/L	538 b	0.912 c	3.40 c	0.317 c	0.196 c	6.58 b
CSP, 10 g/L	1055 a	1.30 b	4.82 b	7.46 b	0.472 b	6.78 b
CSP, 20 g/L	1408 a	1.98 a	5.59 a	19.9 a	0.743 a	8.19 a

[a]A complete medium control ($n = 3$) produced 415 µg of aflatoxin, 0.848 g mycelium, and had a final culture pH of 5.50.
[b]Mean AFB_1 is expressed in µg per flask ($n = 3$). Values in the same column followed by the same letter are not significantly different ($P = 0.05$) by Fisher's LSD.
[c]Mean biomass (dry weight of mycelium) is expressed in grams per flask ($n = 3$).
[d]Mean final pH of culture medium ($n = 3$). See Table 1 for abbreviations.

When employed as the sole nitrogen source, CSP supported aflatoxin production in the defined medium containing sucrose (without nitrate). Aflatoxin production increased with CSP concentration, with cultures containing 20 g CSP per liter forming three times more toxin than those grown in the standard defined medium (with 0.3% $NaNO_3$) (Table 2). Both biomass (dry weight, zein:$r^2 = 0.996$; CSP[–N]:$r^2 = 0.97$, CSP [–C/N]:$r^2 = 0.98$, $P < 0.05$) and final culture pH (zein: $r^2 = 0.87$; CSP[–N]:$r^2 = 0.88$, CSP[–C/N]:$r^2 = 0.94$, $P < 0.05$) were also directly correlated with initial protein concentration (Fig. 1, Table 2). Zein also supported increased aflatoxin production (eight times the defined medium level at 17 g/L) when employed as the sole nitrogen source (Fig. 2). In these cultures aflatoxin increased with culture age through day seven. In contrast, aflatoxin content increased for 11 d in cultures grown in a medium containing zein (17 g/L) as the sole carbon and nitrogen source (Fig. 2).

Effects of zein and CSP on protease production in culture were monitored with an elastin-based radial diffusion assay (4). Cultures with sucrose, with or without protein, produced little or no protease activity. However, cultures with zein or CSP as the sole carbon and nitrogen source produced increased levels of protease activity (2.5 cm^2 with CSP of 1–20 g/L). The peak zein concentration for induction of enzyme production was 2 g per liter. Gel analysis confirmed that protease levels were largely independent of medium CSP concentration (Fig. 3). Denaturing PAGE revealed that the predominant protein in the culture filtrates with protease activity was a 35 kD protein. This protease has been purified and characterized from *A. flavus* previously (5). Increased culture age (zein substrate) resulted in increased protease levels (35 kD protein) (Fig. 4).

DISCUSSION

Stimulation of aflatoxin production by relatively low levels of protein has interesting implications for fungal contamination

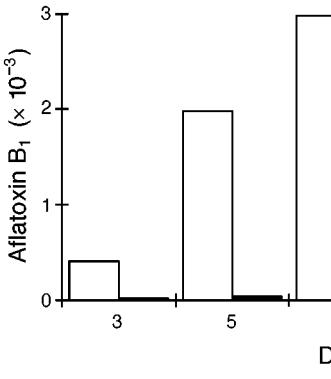

FIG. 2. Effect of culture age on aflatoxin production (µg/flask) in the defined medium without $NaNO_3$ (open bars), and without either sucrose or $NaNO_3$ (closed bars). All incubations (except controls) were supplemented with zein (17 g/L). Defined medium controls were incubated for 5 d and produced 264 µg of aflatoxin ($n = 3$).

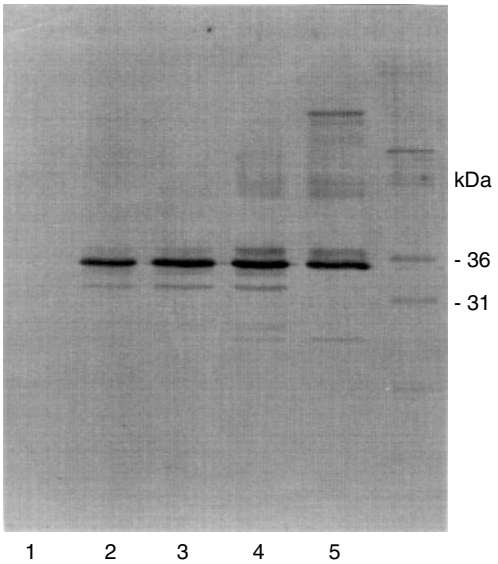

FIG. 3. Effect of cottonseed storage protein (CSP) concentration on *Aspergillus flavus* AF13 protein profile (–C/N condition). Lane 1: Control (defined medium); lane 2: CSP, 1 g/L; lane 3: CSP, 2 g/L; lane 4: CSP, 5 g/L; lane 5: CSP, 10 g/L.

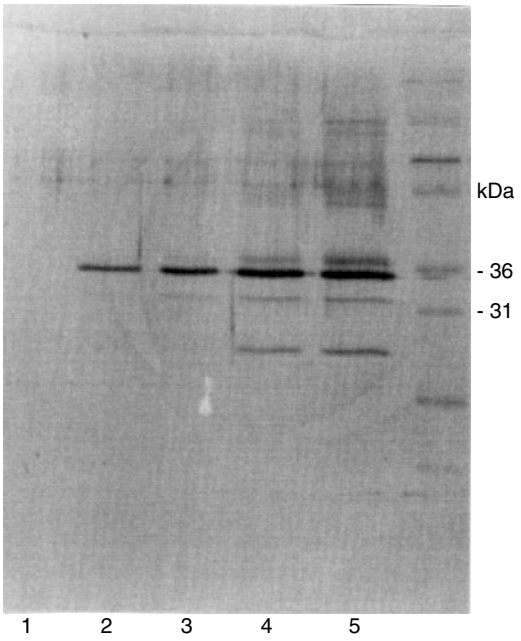

FIG. 4. Effect of culture age on *A. flavus* AF13 protein profile (-C/N condition; zein supplement). Lane 1: 3 d; lane 2: 5 d; lane 3: 7 d; lane 4: 9 d; lane 5: 11 d. See Figure 3 for abbreviation.

of oilseed crops. Toxin stimulation occurred with additions of oilseed proteins (CSP, zein) to the defined medium. There were quantitative differences among the different protein types (Table 1). Of the tested proteins, zein and CSP caused the greatest toxin stimulation. Thus, protein composition of a potential fungal C/N resource can quantitatively influence aflatoxin production. Qualitative alterations in seed protein composition could result in reductions in crop vulnerability to aflatoxin contamination. Plant biotechnology may provide a means to alter amino acid composition of seed storage protein in order to reduce crop susceptibility to contamination.

Use of storage protein as the sole C/N source resulted in reduced toxin production as compared to controls that received sucrose and nitrate. However, reduced toxin production would seem an expected result, considering the level of protein substrate provided (5 g/L) was one-tenth the level of carbon substrate (50 g/L sucrose) available to cultures grown in complete defined medium. *Aspergillus flavus* presumably has a small reserve of protease that is secreted in order to digest the available substrate. Protein is a complex substrate, compared to sucrose, and more time may be required to release carbon into the primary metabolic pools. A high metabolic priority for the fungus would be the production and secretion of additional protease molecules to appropriate additional C/N resources. Collagen was the only protein addendum that yielded aflatoxin levels equivalent to the complete defined medium when used as the sole C/N source. Apparently, this protein can more rapidly replenish secondary metabolite pools to allow for aflatoxin biosynthesis. Biomass production (mycelial dry weight) for the collagen cultures (–C/N) was significantly less than –C/N cultures with other proteins; thus, stimulation of aflatoxin production by collagen is not simply a direct result of increased fungal growth. For zein and CSP, both aflatoxin and biomass production were positively correlated with initial protein concentration. Thus, protein concentration may be an important parameter for aflatoxin production.

The culture age study provided some insight into parameters controlling aflatoxin production. When zein was the sole N source in medium containing sucrose, toxin production was greatly stimulated (eightfold) above defined medium controls (Fig. 2). Apparently, carbon was the limiting resource for toxin production in media where zein was both the C and N source.

Fungal protease activity induced by zein and CSP was the metalloprotease previously purified from *A. flavus* (5). This protease can hydrolyze a wide variety of protein substrates and apparently is the predominant protease activity secreted in order to assimilate carbon from protein substrates. Metalloprotease production was very sensitive to carbohydrate substrate levels. When sucrose was present in the medium, little or no protease was observed. Similar observations have been reported by Srinivasan and Dhar (14) for an uncharacterized protease from *A. flavus*. They also report that cultures actively secreting protease promptly cease upon the addition of a carbohydrate. Carbohydrates appear to repress protease production. With the 35 kD metalloprotease of *A. flavus*, enzyme production was a function of both protein presence (Fig. 3) and culture age (Fig. 4). In the current study, CSP was superior to zein in stimulating protease secretion. Srinivasan and Dhar (14) reported that of the 10 proteins tested for protease production, cottonseed protein stimulates the highest levels.

Culture pH increased with initial protein concentration when either zein or CSP were the sole C and N sources, or when they were the sole N source (sucrose present). Increased pH generally decreases aflatoxin production in *A. flavus* (10) and *A. parasiticus* (15). This observation suggests that a potential greater than that observed may exist for stimulation of aflatoxin biosynthesis by storage proteins. Under conditions where seed tissue homeostasis resists pH increases, storage proteins may stimulate even greater toxin increases.

Carbon sources that are readily accessible to the primary metabolic pathways (e.g. glycolysis) generally support good growth and aflatoxin production in *A. flavus* (16) and *A. parasiticus* (17,18). Recent work suggests that fungal amylase is critical for the induction of aflatoxin production when maize is colonized by *A. flavus* (19). Amylase hydrolyzes starch to produce glucose that appears to initiate toxin production. Maximal aflatoxin production in *A. parasiticus* (20) or *A. flavus* (21) appears to require the presence of a nitrogen source derived from proteinaceous origins. This observation may explain the stimulation of aflatoxin production by protein supplementation of the complete defined medium or by storage proteins serving as the sole N source.

The current results contribute to our understanding of interactions of mycotoxigenic fungi with oilseed crops. A quickly accessed carbon source and a proteinaceous nitrogen source seem to be required for high levels of aflatoxin production in oilseeds. The current data clearly show that if

oilseed storage proteins are the sole carbon/nitrogen source for the fungus, depressed toxin production results. Aflatoxin stimulation by storage proteins in the presence of carbohydrate may occur in both developing corn and cottonseed. In corn, simultaneous hydrolysis of starch and protein bodies may result in conditions similar to the cultures described herein. Similarly in developing cottonseed, lipids present in the developing cotyledons (location of storage reserves) may provide easily accessible carbon. Mature cottonseed contains up to 10% raffinose by weight (22); this storage trisaccharide may also drive aflatoxin production when associated with CSP as in cottonseed. Overall close proximity of storage proteins to accessible carbon resources (carbohydrate or oils) may explain the copious production of aflatoxin in developing oilseeds under certain conditions.

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