Elaboration of Aflatoxin on Cottonseed Products by *Aspergillus flavus*

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Abstract

In controlled laboratory experiments heat sterilized and unautoclaved glanded and glandless whole cottonseed or decorticated kernels and sterilized cottonseed meals were found to be utilized as substrates by an aflatoxin elaborating strain of *A. flavus* with the production of high levels of aflatoxins B_1, B_2, G_1, and G_2. Gossypol pigments in cottonseed products are apparently not a barrier to either mold invasion or aflatoxin production. Cottonseed hulls, lint cotton, and cottonseed linters were found to be poorly utilized as substrates for either mold growth or aflatoxin production.

AFLATOXINS, A COLLECTIVE term describing a mixture of highly toxic and carcinogenic metabolites of some strains of *Aspergillus flavus* Link, were first isolated from moldy peanut meal (1). From this mixture, four compounds designated as aflatoxins B_1, B_2, G_1, and G_2 were subsequently isolated, structurally characterized, and evaluated for biological activity (2–4). These four aflatoxins are presently believed to be primarily responsible for the toxic properties attributable to aflatoxins (4).

Since the original recognition of the aflatoxin problem as one of mold contamination (1), several investigators have reported experimental data on the production of aflatoxins by toxin elaborating strains of *A. flavus* growing on a number of agricultural products in controlled conditions. Codner et al. (5) reported total aflatoxin levels ranging from 14–265 mg per kilograms of substrate when 6 strains of *A. flavus-oryzae* were cultured separately on sterilized peanuts. Although total aflatoxin production varied with strains, the relative proportion of B_1:B_2:G_1:G_2 (40:1:50:1) were found to be reasonably constant. Armbrecht and others (6) demonstrated that high aflatoxin levels were produced when a number of *A. flavus* strains were grown on wheat, buckwheat, oats, corn, rice, soybeans, and peanut meal under moisture and temperature conditions favorable for mold growth, although absolute aflatoxin levels in relation to substrate were not reported. More recently Hesseltine et al. (7) have reported that sorghum, peanuts, soybeans, corn, wheat, and rice not only supported growth of three aflatoxin elaborating *A. flavus* strains in both still and shaken cultures, but produced appreciable amounts of aflatoxins. Both total aflatoxin levels and the ratio of B_1:B_2:G_1:G_2 varied with substrate and mold strain.

Although the experimental evidence clearly indicates that under moisture and temperature conditions favorable for mold growth, many agricultural products can be utilized as substrates by toxin producing strains of *A. flavus*, somewhat conflicting data have been reported in the case of cottonseed products. Thus while Scrimshaw (8) suggested that little if any aflatoxin was produced by *A. flavus* strains growing on cottonseed meal, Loosmore et al. (9) reported toxic levels of aflatoxin B_1 in a sample of cottonseed cake from the Sudan. All four aflatoxins were subsequently detected in a subsample of this cottonseed cake, while only aflatoxins B_1 and B_2 were found in several contaminated samples of domestic cottonseed or cottonseed meals (10). Since cottonseed contains a unique polyphenolic plant pigment, gossypol, in concentrations ranging from about 0.4–1.5% of the weight of the kernel (11), it is possible that this pigment can modify aflatoxin production by *A. flavus* strains. Accordingly laboratory experiments were designed in which normal glanded and glandless (gossypol-free) cottonseed were used as substrates for a toxin producing strain of *A. flavus*. Additionally, cottonseed meals, kernels, lint cotton, and linters were evaluated as substrates. Shredded wheat and shelled peanuts were used as positive controls. Both production of total aflatoxins and the relative proportions of the individual toxins as a function of the substrate used is the subject of the present communication.

Experimental

Substrates

As positive controls, bite-sized, whole shredded wheat biscuits and shelled unblanched Spanish peanuts were used. The glanded cottonseed was prime quality mechanically delintered seed (1964 crop), of unknown variety. Portions of the seed were cracked and screened to provide undamaged kernels and kernel-free hulls. Glandless cottonseed was an experimental Acala strain (1962 crop) field pollinated and stored at low temperature. The seed was essentially free of gossypol pigments. The cottonseed meals were a commercial screw-pressed meal and experimental meal extracted with an acetone:hexane:water mixture (12). Lint cotton, surgical cotton and first cut cottonseed linters were of commercial origin. Both lint cotton and surgical cotton were used with and without addition of essential trace metals and a nitrogen source.

A. flavus Strain

The strain used was originally isolated from peanut meal by British workers who labeled it 3734/10. A subculture, M-3, was supplied by the US Food and Drug Administration (6). Other subcultures of this strain were employed by Codner et al. (5), Armbrecht (6), and Hesseltine (7). The strain was kept in stock on potato dextrose agar. Inocula of approximately 3 million spores per milliliter in 0.05% Tween 20 solution were prepared from growth of the mold on potato dextrose agar plates incubated at 30C for 7 days.

Conditions of Aflatoxin Production

Substrate samples of 25 g were weighed into sterile, cotton-plugged 1 liter Erlenmeyer flasks, and sterile tap water was added to a calculated moisture level of 33%. Where sterilized substrates were desired, the flasks containing the materials and tap water were

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autoclaved under 15 lb steam pressure for 15 min and the moisture loss was replaced with sterile distilled water. All substrates were inoculated with 2 ml of spore suspension and incubated at 30C for 7 days. After visual observation and notation of growth, the mold was killed by refluxing with chloroform in the flasks for 5 min on a steam bath. Excess chloroform was removed by gentle evaporation before analysis for aflatoxin content. Duplicate experiments performed several weeks apart were conducted in most cases.

Aflatoxin Analysis

The entire chloroform treated and evaporated samples were quantitatively transferred to 1 liter Waring Blendor jars with 250 ml of acetone:water (70.30 v/v) and the contents were blended at high speed using an explosion-proof blender. The crude extracts were filtered through paper, and 150 ml aliquots were analyzed for aflatoxins by the procedure described by Pons and Goldblatt (10). Final extracts from the analytical procedure were suitably diluted with chloroform, usually 1:1000 to 1:5000, to provide solutions for TLC analysis.

Results and Discussion

Concentrations of aflatoxins produced on sterilized substrates are tabulated in Table I. Total aflatoxin production substantially equivalent to that produced on shredded wheat, and over twice the levels produced on peanuts, were obtained with sterilized glanded or glandless seed, and kernels (Table I). Both the total aflatoxin levels and the ratios of B_1:G_1 were relatively constant, while somewhat greater variability was found for the ratio of B_1:B_2. The lowest aflatoxin levels and B_1:B_2 ratios were produced on the cottonseed meals. Aeration has been suggested by Hesseltine (7) as a critical variable in aflatoxin production, and these lower aflatoxin levels may have resulted from caking of finely ground meals and reduced aeration rates.

When unautoclaved substrates were employed (Table II), high and essentially equivalent total aflatoxin levels were again produced on both glanded and glandless whole seed, suggesting that gossypol pigments in glanded seed have no apparent influence on aflatoxin elaboration by the *A. flavus* strain used. Substantially lower levels of total aflatoxins were produced on the unautoclaved kernels than on the whole cottonseed. This trend was observed in three separate experiments and the differences in aflatoxin contents of the whole seeds and kernels were much larger than could be attributed to variations in experimental technique.

TABLE I

Aflatoxin Elaboration on Autoclaved Substrates Inoculated with *A. flavus*

Substrate	Mold growth [a]	Aflatoxins found mg per kg					Ratio	
		B_1	B_2	G_1	G_2	Total	B_1:B_2	B_1:G_1
Shredded wheat	++	720	60	200	20	1000	12:1	3.6:1
Shelled peanuts	++	250	30	160	20	460	8.3:1	1.6:1
Glanded whole c.s. [b]	++	440	70	430	70	1010	6.3:1	1.0:1
Glanded c.s. kernels	++	510	70	370	80	1030	7.3:1	1.4:1
Glandless whole c.s.	+	690	160	490	110	1450	4.3:1	1.4:1
Glandless c.s. kernels	++	480	70	490	90	1130	6.9:1	1.0:1
Screw-press c.s. meal [c]	++	110	40	140	40	330	2.8:1	0.8:1
Solvent extd. c.s. meal [d]	++	100	40	130	30	300	2.5:1	0.8:1

[a] Estimated by appearance only:
+ = Some, but not all seeds with spores
++ = Covered with spores.
[b] c.s. = cottonseed.
[c] Commercial meal.
[d] Extracted with acetone:hexane:water mixture.

TABLE II

Aflatoxin Elaboration on Live Substrates Inoculated with *A. flavus*

Substrate	Mold growth [a]	Aflatoxins found, mg per kg					Ratio	
		B_1	B_2	G_1	G_2	Total	B_1:B_2	B_1:G_1
Shelled peanuts	+	300	50	170	30	550	6:1	1.8:1
Glanded whole c.s. [b]	+	760	230	570	150	1710	3.3:1	1.3:1
Glanded c.s. kernels	++	170	15	130	10	325	11:1	1.4:1
Glandless whole c.s.	±	680	110	430	60	1280	6.2:1	1.6:1
Glandless c.s. kernels	++	350	30	210	40	630	12:1	1.6:1

[a] Estimated by appearance only:
± = A few seeds with spores
+ = Some, but not all seeds with spores
++ = Covered with spores.
[b] c.s. = cottonseed.

It may be noted that all but three of the cottonseed samples listed in Tables I and II were found to be covered with a heavy growth of dark green *A. flavus* spores. However, in the three samples where observable mold growth was not as heavy, high levels of aflatoxins were nevertheless found. It must be concluded that the outward appearance of mold growth is not a reliable index of the amount of aflatoxins elaborated by *A. flavus*. Also, since two of these samples and a shelled peanut control (Table II) can be assumed to be living at the beginning of the test, this conditoin did not protect from production of aflatoxin under the adverse conditions.

In the experiments reported here, in which the English isolate of a strain of *A. flavus* was used, the ratios of aflatoxin B_1:B_2 ranged from about 2.5:1 to 12:1, while the ratios of B_1:G_1 were relatively constant, 0.8:1 to 1.6:1. In contrast to these results, analyses made in this laboratory of some 40 naturally contaminated domestic cottonseed and cottonseed meal samples indicated the presence of primarily aflatoxins B_1 and B_2, and these were in relatively constant ratios, ranging from 1:1 to 3:1 with an average value of 2.5:1. Such results suggest that the infrequent occurrence of aflatoxins G_1 or G_2 in these domestic cottonseed products may be attributable more to the characteristics of the individual mold strains present than to any inhibitory effect of cottonseed constituents.

Although both whole cottonseed and isolated kernels were utilized by the *A. flavus* strain to produce high aflatoxin levels, the data presented in Table III suggest that cottonseed hulls and cotton cellulose are poorly utilized as substrates, both for mold growth and for aflatoxin elaboration. It may be noted that production of total aflatoxins on the autoclaved constituent parts of the cottonseed is in decreasing order of kernels:hull:lint at approximate ratios of 1000:100:1.

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TABLE III

Aflatoxin Elaboration on Autoclaved Cellulosic Materials Inoculated with *A. flavus*

Substrate	Mold growth [a]	Aflatoxins found mg per kg					Ratio	
		B_1	B_2	G_1	G_2	Total	B_1:B_2	B_1:G_1
c.s. [b] hulls	—	60	1	50	1	112	60:1	1.2:1
Lint cotton	—	0.32	0.06	0.16	0.02	0.56	5:1	2:1
Lint cotton + salts [c]	—	0.44	0.06	0.32	0.05	0.87	7:1	1.4:1
c.s. linters	—	0.38	0.13	0.21	0.11	0.83	3:1	1.8:1
Absorbent cotton	—	0.38	0.13	0.21	0.05	0.77	3:1	1.8:1
Absorbent cotton + salts	—	1.27	0.57	0.64	0.32	2.80	2:1	2:1
Inoculum only	[d]	0	0	0	0	0	—	—

[a] Estimated by appearance only:
— = No spores or mycelium distinguishable.
[b] c.s. = Cottonseed.
[c] Salts: Essential trace metals and nitrogen source as used in Czapek's agar (13).
[d] About 6 million spores.

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