Extraction and Separation of the Bright-Greenish-Yellow Fluorescent Material from Aflatoxigenic *Aspergillus* spp. Infected Cotton Lint by HPLC−UV/FL

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To isolate the bright-greenish-yellow-fluorescence (BGY-F) material associated with aflatoxin contamination on cotton lint, various in vitro chemical and in vivo natural BGY-F reaction products were prepared. BGY-F material was obtained from reactions involving (1) kojic acid (KA) and H_2O_2, (b) KA, peroxidase, and H_2O_2, (c) fresh cotton locules treated with KA and H_2O_2, (d) detached cotton locules inoculated with an aflatoxigenic *Aspergillus flavus* spore suspension, and (e) live developing cotton bolls inoculated with aflatoxigenic *A. flavus*. BGY-F preparations separated on TLC resulted in the same R_f value (0.52), indicating the possibility of the same compound. Under HPLC conditions, at a 380 nm wavelength setting, BGY-F retention times ranged 5.74−6.09 min; under fluorescent detection at 435 nm (excitation) and 494 nm (emission), retention times ranged from 5.75 to 6.09 min. Apparently, in all methods used to form the BGY-F compound, both in vivo and in vitro, only one compound with specific chromatographic characteristics was produced, and the product is likely an oxidized form of KA.

Keywords: *Cotton; Gossypium hirsutum L.; bright-greenish-yellow fluorescence (BGY-F); Aspergillus flavus; aflatoxin; HPLC−UV/F*

INTRODUCTION

Aflatoxins are polyketide-derived secondary metabolites produced by the aflatoxigenic fungi *Aspergillus flavus* Link ex. Fries and *Aspergillus parasiticus* Speare (Detroy et al., 1971). These metabolites (particularly aflatoxin B_1) are not only toxic to animals and humans but are the most carcinogenic of all known natural compounds (Groopman et al., 1981; Busby and Wogan, 1979; Bosch and Peers, 1991; Hall and Wild, 1994). Aflatoxin contamination in cottonseed (*Gossypium hirsutum* L.) can drastically decrease the economic value of the crop and can present a medical threat to both man and animal.

It is reported that aflatoxigenic *Aspergillus* invasion of the developing cotton boll results in the formation of a characteristic bright-greenish-yellow-fluorescent (BGY-F) material when the cotton lint of the infected boll is viewed under long wave UV light (Marsh et al., 1955). It is well established (Marsh et al., 1973) that BGY-F results from the reaction of host plant peroxidase with the fungal metabolite, kojic acid (KA). KA (5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one), the precursor of the BGY-F material, is produced by a limited number of *Aspergillus* spp. but is produced by both the aflatoxigenic *Aspergillus* spp., *A. flavus* and *A. parasiticus* (Parrish et al., 1966). It has also been demonstrated that this fluorescence compound is formed only on the lint in the developing cotton boll while aflatoxin is formed exclusively in the seeds (Marsh et al., 1973; Lee and Russell, 1981). In vitro studies have shown that the BGY-F material can be obtained in solutions of peroxidase, hydrogen peroxide, and KA (Marsh et al., 1969). KA and BGY-F appear to be closely related, but their relationship and mechanism of formation is limited and mostly unknown.

A procedure was developed by Lillehoj et al. (1986) to extract and estimate the BGY-F material from corn and Kitao and Sekine (1994) reported the separation of KA and its transglucosylation products by HPLC. However, more effective methods are needed for the separation and purification of BGY-F and its coproducts from cotton boll associated with aflatoxin contamination before attempts of chemical identification proceed.

The purpose of this present investigation was to determine if the in vivo formed BGY-F material and the natural product BGY-F material result in similar HPLC chromatographic characteristics. The purpose also was to develop an HPLC system to purify both the in vitro product and the natural product from substances that would interfere with the identification of the BGY-F product.

MATERIALS AND METHODS

Reagents. Kojic acid, peroxidases from horseradish (HRP), type VI-A and type II, peroxidases from soybean (SBP), guaiacol (2-methoxyphenol), and hydrogen peroxide (31.1%) were purchased from Sigma Chemical (St. Louis, MO). Sodium hypochlorite solution with 5% available chlorine was purchased from Aldrich Chemical, Inc. All other chemicals were of reagent grade. TLC plates (precoated cellulose, 0.01 and 0.5 mm thick without fluorescent indicator) were purchased from EM Reagents Co. Clorox (The Clorox Co., Oakland, CA) containing 5.25% sodium hypochlorite was used to disinfect developing cotton bolls.

Fungal Strain and Culture Conditions. An aflatoxigenic isolate of a wild-type strain of *A. flavus* (SRRC 1000A) obtained from Arizona cottonseed was cultured on potato

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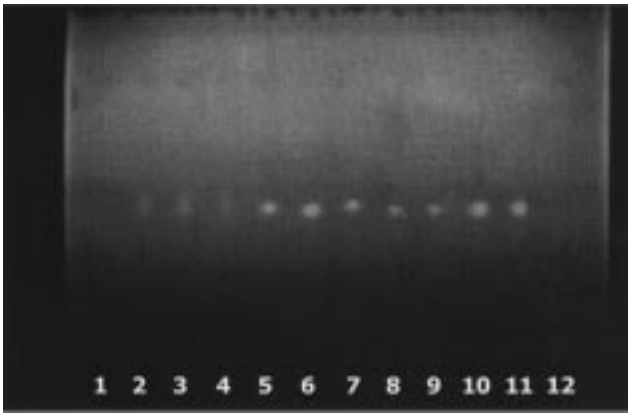

Figure 1. Photograph taken under UV light at 366 nm. TLC separation of KA and BGY-F reaction products by various methods: (1) KA, 750 ppm; (2) KA + peroxidase (from soybean) + H_2O_2; (3) KA + HRP type VI-A + H_2O_2; (4) KA + HRP type II + H_2O_2; (5) KA + NaOCL + H_2O_2; (6) KA + cotton locules + H_2O_2′; (7) *A. flavus* + cotton locules, laboratory; (8) *A. flavus* + cotton locules, field; (9) KA + NaOCl + H_2O_2, purified by TLC; (10) KA + HRP type II + H_2O_2, purified by TLC; (11) KA + HRP type VI-A + H_2O_2, purified by TLC; (12) KA, 750 ppm.

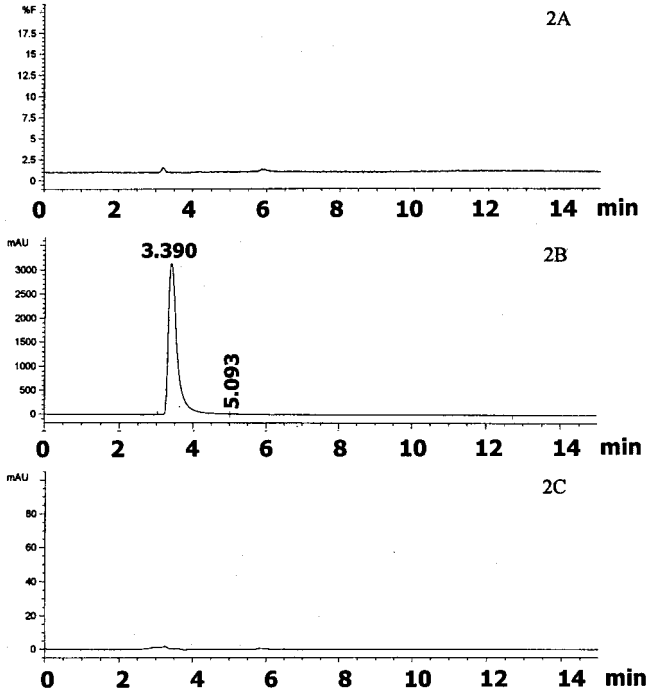

Figure 2. HPLC chromatograms of KA, 750 ppm, with fluorescent detector at excitation 435 nm, emission 494 nm (A) and with UV detector at 280 nm (B) and at 380 nm (C).

dextrose agar Petri plates; spores were extracted from plates for spore suspension preparations utilized in infecting developing cotton bolls.

Cotton Plants and Conditions. Acala SJ-2 cotton plants were grown in separate experimental plots in the field at the Southern Regional Research Center in New Orleans, LA. At 20−32 days postanthesis, injections of spore suspensions were made directly into the bolls in experiments on the intact bolls or on detached bolls for the various locule treatments.

Preparation of BGY-F Reaction Products. *Reaction Product 1* (*Production of BGY-F from KA and H_2O_2).* A 0.1 mL aliquot of 31.1% H_2O_2 and 0.5 mL of NaClO were added dropwise over a 20 min time period into a KA solution (25 mg/20 mL of H_2O). After 3 h, the reaction mixture was lyophilized and stored in the dark at 4 °C.

Reaction Product 2 (Production of BGY-F from KA in the Presence of Peroxidase and H_2O_2). A 1.0 mg sample of peroxidase (SBP, HRP, types VI-A and II) and 500 mg of KA were added to 100 mL of a 0.0003% H_2O_2 solution. The mixture was incubated at room temperature in the dark for 4 h; peroxidase was removed with a Centriprep-30 Concentrator (molecular cutoff weight of 30 000). The solution was lyophilized and stored in the dark at 4 °C.

Reaction Product 3 (Production of BGY-F from Fresh Locules from Cotton Bolls That Were Treated with KA and H_2O_2). The peroxidase presence was tested in fresh cotton boll locules with a guaiacol test solution. Locules were soaked with the guaiacol

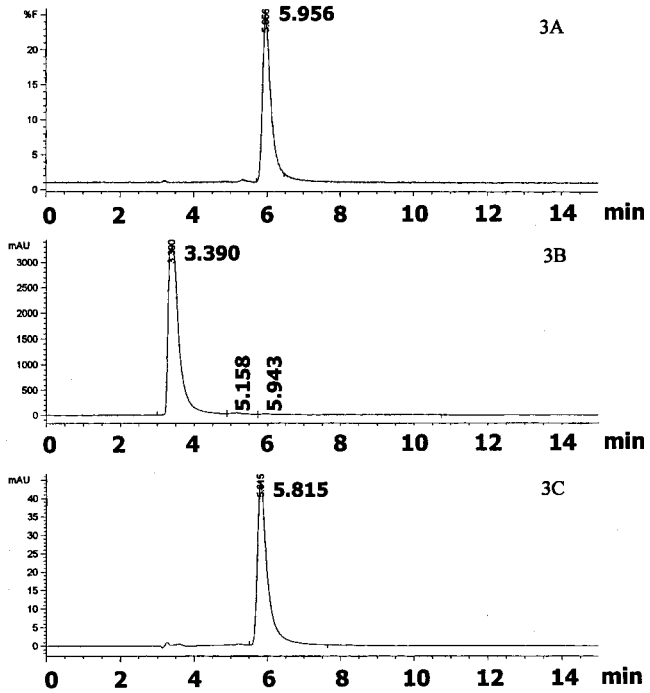

Figure 3. HPLC chromatograms of BGYF and other products from reaction product 2 (HRP type II), with fluorescent detector (A) and with UV detector at 280 nm (B) and at 380 nm (C).

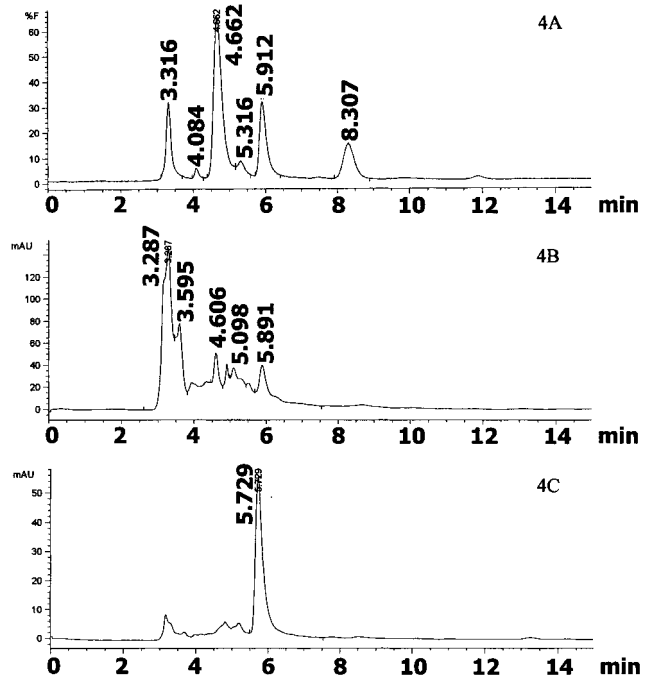

Figure 4. HPLC chromatograms of BGY-F and other products from reaction product 5, with fluorescent detector (A) and with UV detector at 280 nm (B) and at 380 nm (C).

Table 1. Summary of HPLC Retention Times (min) of Kojic Acid (KA) and Various BGY-F Reaction Products by Variable Wavelength (VW) Detection (280 nm) and by Fluorescent (FL) Detection (435 nm Excitation, 494 nm Emission, 450 nm Filter)

reacn product	reacn conditions	retention times (min)	
		VW detection	FL detection
	kojic acid (KA)	**3.39**, 5.09	
1	KA + NaOCl + H_2O_2	**3.38**, 5.54, **6.04**, 9.89, 13.29	5.54, **6.05**
2	KA + SBP[a] + H_2O_2	**3.39**, 5.16, **5.95**	**5.96**
2	KA + HRP[b] type II + H_2O_2	**3.39**, 5.16, **5.94**	**5.96**
2	KA + HRP type VI + H_2O_2	**3.37**, 5.12, **5.92**	**5.93**
2	KA + HRP type II, TLC purification[3]	3.18, 3.43, 5.12, 5.51, **6.05**	3.30, 5.37, **6.09**
1	KA + NaOCl + H_2O_2 TLC[a] purification	3.44, 5.15, **6.22**	3.22, 5.15, **6.23**
2	KA + HRP type VI, TLC purification	3.18, 3.44, 5.16, **6.03**	3.29, 5.37, **6.04**
3	KA + locules + H_2O_2	**3.39**, 4.85, 5.13, **5.87**	3.31, 4.82, **5.92**
4	*A. flavus* + locules, laboratory	**3.42**, 5.13, 5.37, **5.92**	3.31, 4.76, **5.91**
5	*A. flavus* + locules, field	**3.29**, 3.60, 4.61, 5.10, **5.89**	3.32, 4.08, 4.66, 5.32, **5.91**, 8.31

[a] Peroxidases from soybean. [b] Peroxidases from horseradish. [c] TLC = BGY-F reaction products were prepurified by separations on precoated cellulose plates (0.5 mm), developed in a mixture of formic acid−methyl ethyl ketone−*tert*-butyl alcohol−water (3:6:8:3).

Table 2. Summary of HPLC Retention Times (min) of Kojic Acid (KA) and Various BGY-F Reaction Products by Variable Wavelength (VW) Detection (380 nm) and by Fluorescent (FL) Detection (435 nm Excitation, 494 nm Emission, 450 nm Filter)

reacn product	reacn conditions	retention times (min)	
		VW detection	FL detection
	kojic acid (KA)		
1	KA + NaOCl + H_2O_2	6.00	**6.00**
2	KA + SBP[a] + H_2O_2	5.85	**5.86**
2	KA + HRP[b] type II + H_2O_2	5.82	**5.82**
2	KA + HRP type VI + H_2O_2	5.74	**5.75**
2	KA + HRP type II, TLC purification[c]	5.91	3.33, 5.24, **5.94**
1	KA + NaOCl + H_2O_2, TLC purification[c]	6.09	3.23, 5.08, **6.09**
2	KA + HRP type VI, TLC purification[c]	5.87	3.32, 5.25, **5.88**
3	KA + locules + H_2O_2	5.76	3.32, 4.72, **5.77**
4	*A. flavus* + locules, laboratory	5.75	3.32, 4.71, **5.76**
5	*A. flavus* + locules, field	5.73	3.33, 3.99, 4.72, 5.21, **5.74**, 7.89

[a] Peroxidases from soybean. [b] Peroxidases from horseradish. [c] TLC = BGY-F reaction products were prepurified by separations on precoated cellulose plates (0.5 mm), developed in a mixture of formic acid−methyl ethyl ketone−*tert*-butyl alcohol−water (3:6:8:3).

test solution, and 1−2 drops of a 3% H_2O_2 solution was added. After 5 min, a brown color formed, indicating the presence of peroxidase. Twenty cotton locules were soaked overnight in 40 mL of a 0.1% KA solution (w/v) containing 400 μL of a solution of 31% H_2O_2. The fluorescent solution was collected by filtering with Miracloth (Calbiochem-Novabiochem Corp., LaJolla, CA). The fluorescent materials on the lint in the locules were extracted three times with H_2O, combined, and then lyophilized. The brownish-yellow product was stored in the dark at 4 °C.

Reaction Product 4 (Production of BGY-F from Detached Cotton Locules Inoculated with an A. flavus (SRRC 1000A) Spore Suspension). Ten freshly picked cotton bolls (20−32 days postanthesis) were disinfected by soaking in 10% Clorox solution for 2 min and rinsing with sterilized water three times. The locules were separated from the capillary wall and intercapillary membrane manually by using a sterilized knife. The locules were placed in a sterilized glass Petri dish with a layer of small glass beads. A 20 mL of aliquot sterilized water was added to cover the beads, and 20 μL of *A. flavus* 1000A (3.0×10^6 spores/mL) spore suspension solution was injected into each locule by a syringe. The dish was covered and sealed with parafilm and stored in the temperature control chamber (28 °C) for 20 days. The locules were examined under the wavelength ultraviolet light, the BGY-F containing fibers were separated manually, and the BGY-F material was extracted from cotton fiber with water and lyophilized.

Reaction Product 5 (Production of BGY-F from Live Developing Cotton Bolls That Had Been Inoculated with A. flavus (SRRC 1000A)). The developing cotton bolls (20−32 days postanthesis) were each inoculated with 20 μL of an *A. flavus* spore suspension (3.0×10^6/mL). Two weeks after inoculation, the cotton bolls were harvested and examined under the long-wavelength ultraviolet light. The cotton lint containing BGY-F material was extracted with water and lyophilized.

TLC Separation of KA and the BGY-F Reaction Products. KA and BGY-F products were spotted on a precoated cellulose plate and were developed in a solution mixture of formic acid−methyl ethyl ketone−*tert*-butyl alcohol−water (3:6:8:3). The plate was examined under long- (366 nm) and short-wavelength (254 nm) ultraviolet light (Ultra-Violet products, Inc.). The R_f values of the various BGY-F reaction products were calculated. Figure 1 is a photograph of a typical TLC plate.

Each of the three reaction products consisting of KA and BGY-F including KA + NaOCL + H_2O_2, KA + HRP (VI) + H_2O_2, and KA + HRP (II) + H_2O_2 was spotted on a precoated cellulose plate and developed in the eluting solution mentioned above. The BGY-F band was scraped off the TLC plate, and the dried cellulose powder was extracted five times with H_2O, filtered, and then lyophilized.

HPLC Separation of KA and BGY-F Reaction Products. Separations were conducted on a Hewlett-Packard (HP) liquid chromatograph equipped with an HP Model 1050 pump, an HP 1046 AX programmable fluorescence detector, an HP G1314A variable wavelength detector, and an HP Chemstation data acquisition and integration software system. The UV detector was set at 280 and 380 nm, and the fluorescence detector was set at an excitation setting of 435 nm and an emission setting of 494 nm and measured with a 450 nm cutoff filter.

An Alltech Econosphere NH_2 column (250 × 4.6 mm i.d.; particle size, 5 μm) was used for chromatographic separations. The column system included an Alltech guard column packed with Econosphere NH_2. Chromatographic peaks were recorded and integrated with an HP Chemstation automation system. Twenty microliters of reaction product was injected, and isocratic elution was conducted with a flow-rate of 1.0 mL/min.

Elution of KA and BGY-F with 0.05 M potassium phosphate

buffer and methanol (45:55) was completed in 15 min. The eluting solvents were prepared by adding methanol to phosphate buffer, filtered through a 0.45 μm Millipore filter, and degassed continually by the on-line helium degas system.

RESULTS AND DISCUSSION

Initially, various BGY-F preparations were examined by thin-layer chromatography. Under UV light at 366 nm, BGY-F (columns 2−11, Figure 1) showed an intense fluorescent spot in the middle of the TLC plate. Under UV light at 254 nm, the KA showed a black spot in the top section of the TLC plate (columns 1−6, and 12, Figure 1). The R_f values of KA and BGY-F were calculated to be 0.84 and 0.52, respectively. In the photograph, Figure 1, taken under UV light at 300 nm, KA (columns 1 and 12) was faintly recognizable whereas the BGY-F compounds from the various preparations showed an intense spot at almost the identical rate of elution. The BGY-F reaction products produced under various conditions showed the same R_f, value indicating that they might be the same compound.

Successful HPLC separations of KA and BGY-F reaction products were obtained, under isocratic conditions, using an eluting solvent system of 0.05 M potassium phosphate buffer and methanol (45:55). Typical chromatograms of KA and BGY-F are shown in Figures 2 and 3, using KA and products from reaction product 2 (HRP type II). BGY-F was strongly detected with a retention time of approximately 6 min by fluorescent detection (Figure 3A) and UV detection at 380 nm (Figure 3C). KA was detected only at 280 nm with a retention time of approximately 3.4 min (Figures 2B and 3B). BGY-F from reaction product 5 (BGY-F from live developing cotton bolls that had been inoculated with *A. flavus*) was again detected by fluorescence at approximately 6 min (Figure 4A) and at 380 nm (Figure 4C), but KA was difficult to detect at the retention time of 3.4 min, even with the 280 detector, because of many other compounds coeluting at about the same retention time (Figure 4B).

Data of HPLC are summarized in Tables 1 and 2. KA has been reported to have a maximum absorption at 270 nm, whereas the BGY-F reaction product from the reaction of the horseradish peroxidase (HRP)/H_2O_2 system is 375 nm (Kahn et al., 1995). With the UV detector set at 280 nm, Table 1, HPLC separation shows that KA and BGY-F product were eluted at 3.3−3.4 min and 5.9−6.2 min, respectively. A peak at the retention time of 5.9−6.0 min was observed for the BGY-F product by fluorescent detection. With the UV detector set at 380 nm, there is only one peak at 5.9 ± 0.2 min, which is apparently the BGY-F product. KA was not observed in the chromatograms of fluorescence and UV at 380 nm. The major peak on the fluorescent detector chromatogram at 5.9 ± 0.2 min is apparently from the elution of the BGY-F. However, for reasons still unknown, the retention time from the reaction of KA with NaOCl and H_2O_2 was slightly but consistently longer than the 5.9−6.0 min normally shown by BGY-F from other sources (Tables 1 and 2). A possible explanation is that peroxidases ususally form OH free radials from H_2O_2 while hypochlorite forms single oxygen molecules; it is conceivable that the two systems could form slightly different compounds. The three reaction products (KA + NaOCl and H_2O_2, KA + HRP VI + H_2O_2, and KA + HRP II + H_2O_2) after TLC purification show more than one peak in the HPLC chromatogram at 280 nm and fluorescence, suggesting that thin-layer chromatography is not effective in their separation. All reactions using fresh locules (Tables 1 and 2) yielded multifluorescent products. Compounds other than BGY-F could also be produced by the transglucosylation of KA with sugars in cotton locules.

Apparently, in all the methods we used to produce BGY-F, in vitro chemical or in vivo, only one compound with specific chromatographic BGY-F characteristics was produced. Aromatic phenols have been known to be readily oxidized by the HRP/H_2O_2 system (Saunders et al., 1964; Danner et al., 1973; Guilbault et al., 1968). HRP is readily available in the tissues of healthy cotton bolls. The activity of peroxidase reaches its maximum as the bolls approached maturity at about 30 days of age (Wang and Pinckard, 1973). The presence of peroxidase and H_2O_2 in fresh and/or living cotton locules can apparently catalyze oxidation of the KA produced by invading *A. flavus* and form the BGY-F compound. We and others (Kahn et al., 1995) believe that the BGY-F compound is probably an oxidized form of kojic acid, but we do not have any information about its chemical structure.

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