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Fluorometric analysis of iodinated aflatoxin in minicultures of *Aspergillus flavus* and *Aspergillus parasiticus**

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SUMMARY

A convenient miniassay for aflatoxin has been developed for cultures of *Aspergillus flavus* and *A. parasiticus* grown for 3–10 days in 10 ml of a coconut extract medium. The sensitivity of the assay, as measured by photofluorometry (365 nm maximum excitation; 445 nm maximum emission), is of the order of 0.01 μM (3.12 ng/ml) for aflatoxin B_1 dissolved in aqueous iodine (0.26 mM). High performance liquid chromatography, monitored by fluorometric analysis of both an aflatoxin B_1 standard and selected culture filtrates, confirmed the sensitivity of the assay and indicated specificity for iodine-enhanced fluorescence of aflatoxin in the coconut extract medium. Thin layer chromatography further confirmed the aflatoxin titers and the specificity for enhancement of aflatoxins B_1 and G_1 in culture filtrates.

INTRODUCTION

Conventional procedures for the measurement of aflatoxin in both laboratory media and field samples typically involve extraction of these toxins with volatile solvents [1]. A direct photofluorometric analysis of aflatoxin has been developed for use in the study of the genetics and physiology of aflatoxin production by strains of *Aspergillus flavus* and *A. parasiticus*. This procedure has the potential to measure aflatoxin with great sensitivity, permits rapid determination of aflatoxin without the need of solvent recovery, and avoids the use of hazardous and expensive solvents.

MATERIALS AND METHODS

Fungal strains and culture media. Two strains of *Aspergillus flavus* Link (NRRL 6539, 5565) and one strain of *Aspergillus parasiticus* Speare (NRRL 3240) were maintained on slants of potato dextrose agar (PDA, Difco) and are herein designated, respectively, as strains Nos. 1, 2 and 3. Conidia harvested from 2-week-old cultures grown at 25°C were suspended in 10 ml of distilled water containing 0.5% Triton X-100 (Sigma). Suspensions were sonicated for 1–2 min in an ultrasonic cleaner

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(Cole-Parmer, Model 8845-3). Spore titer was determined by haemocytometer counts and viability by dilution on PDA plates.

Each fungal strain was evaluated for aflatoxin synthesis after growth in a coconut extract medium modified from that reported by Davis et al. [5]. The medium was prepared by homogenizing 100 g of grated and dehydrated 'Gem' coconut (Baker, Dover, DE) in 300 ml of distilled water for 5 min at high speed in a blender (Waring, Model 31BL91) followed by filtration through four layers of cheesecloth (Curity). The extract was diluted 1:1 with water and the pH was adjusted to 7.0 with 1 N NaOH prior to autoclaving for 18 min at 121°C. The coconut extract broth (CEB) was placed either in nonfluorescent Pyrex test tubes (150 × 18 mm) stoppered with 'Bacti-Capall' caps to contain 10 ml or in Erlenmeyer flasks (250 ml) stoppered with a silicone sponge closure (Bellco Glass, Vineland, NJ) to contain 100 ml. After inoculation with a predetermined number of spores, test tube cultures were vortexed briefly and incubated as stationary cultures at 25°C for 10 days. These cultures were vortexed momentarily after 3, 5, and 7 days of incubation. Flask cultures were vortexed briefly only at the time of inoculation and incubated without further agitation at 25°C for 7 or 10 days.

Determination of standard curves. Standard curves for fluorescence of aflatoxin B_1 were determined using a commercial standard (Sigma, A-6636) in both the presence and absence of iodine. A previously developed photofluorometric (PFM) procedure [3], optimized for enhancement of fluorescence relative to iodine concentration, was employed. Fluorescence was measured with a Coleman Photofluorometer Model 12C (Perkin-Elmer, Oak Brook, IL) with primary excitation filtration at 365 nm max. and secondary emission filtration at 445 nm max. The aperture on the primary filter was fully open to attain maximum sensitivity. Equipment was identical to that used previously [3] and lacked a digital or expanded scale readout available on current models of such equipment. A standard curve for iodine-enhanced fluorescence of aflatoxin B_1 was also determined by high pressure liquid chromatography (HPLC) following a procedure described earlier [5]. Purified standard was initially dissolved in dimethyl sulfoxide at a concentration of 3.12 mg/ml and diluted in distilled water or in aqueous solutions of iodine (0.026–1.3 mM) to obtain an extended series of standard solutions and/or suspensions of aflatoxin ranging from 0.05 to 10 μM (1.56–3120 ng/ml).

Analysis of aflatoxin standard in CEB. The aflatoxin B_1 standard was added to uninoculated CEB and diluted in aqueous iodine (0.26 mM) to obtain a series of standard aflatoxin solutions at concentrations of 0.1, 1.0, and 10 μM. These were also analyzed by PFM and HPLC to derive standard curves for fluorescence of iodine-enhanced aflatoxin. Samples analyzed by HPLC were first filtered (Zetapor, 25 mm, AMF Cuno Div., Meriden, CT) to remove any particulate material greater than 0.2 μm. Samples analyzed by PFM were similarly filtered or diluted at least 100-fold from the original CEB concentrations to minimize the presence of interfering particles during excitation at 365 nm.

Analysis of aflatoxin in CEB cultures. Replicated samples of 10 ml cultures grown for 3–10 days were analyzed periodically by PFM by removing 0.5 ml of a freshly vortexed culture and adding this aliquot to 4.5 ml of 0.26 mM aqueous iodine. Samples were then serially diluted in the aqueous iodine solution and fluorescence was measured relative to the standard curve determined for aflatoxin B_1 by PFM. Selected samples were filtered as described and analyzed by HPLC and thin layer chromatography (TLC) as confirmatory tests for the determination of titer and the specificity of iodine enhancement of aflatoxin fluorescence. Flask-grown cultures of strains Nos. 1 and 3 were evaluated after 7 and 10 days by direct PFM in aqueous iodine and by conventional TLC [1] following solvent extraction of noniodinated aflatoxin with 150 ml of chloroform.

RESULTS

The enhancement of aflatoxin fluorescence by iodine, determined previously by PFM [3] and confirmed subsequently by HPLC [5], has been adapted procedurally in this research for direct measure-

ment of aflatoxin production by *A. flavus* and *A. parasiticus*. The enhancement phenomenon was first optimized using an aflatoxin B_1 standard dissolved in aqueous iodine solutions ranging from 0.026 to 1.3 mM (Table 1). Optimal enhancement was determined by PFM to occur with 0.26 mM iodine. Comparable enhancement was also observed for the same aflatoxin B_1 standard added first to CEB and diluted serially into 0.26 mM aqueous iodine. These latter solutions of aflatoxin B_1 were also analyzed by HPLC following ultrafiltration to remove particulate materials from CEB, and a standard curve for quantitation of aflatoxin was determined (Fig. 1). The HPLC analysis confirmed that iodine enhancement of fluorescence in the uninoculated medium was specific for the added aflatoxin B_1, since only one peak consistent with the known retention time of iodinated aflatoxin B_1 [5] was observed.

Similarly, a standard curve for aflatoxin B_1 in aqueous iodine (0.26 mM) was developed through PFM analysis (Fig. 2). A standard curve for aflatoxin B_1 fluorescence in the absence of iodine was also determined by PFM (Fig. 3), indicating an approximate 100-fold increase in sensitivity for aflatoxin detection at the optimal level of iodine compared to that for noniodinated aflatoxin B_1. This increase in sensitivity and the availability for aflatoxin production of an efficient medium, which had no apparent interfering fluorescence with iodine addition, made it feasible to examine by direct PFM representative fungal strains for aflatoxin produc-

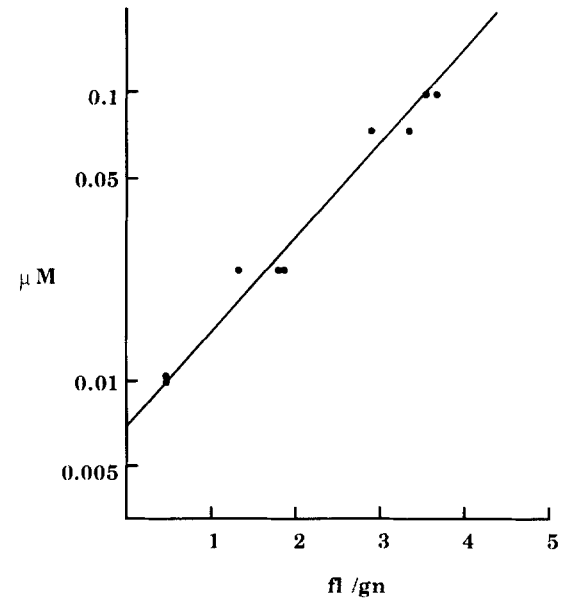

Fig. 1. Standard curve for aflatoxin B_1 (μM) in aqueous iodine determined by HPLC. fl/gn are fluorescence over gain units.

Table 1

Relative enhancement of fluorescence for aflatoxin B_1 in aqueous iodide[a]

Data represent relative units of fluorescence on a scale of 0–100. Recorded values are average of three samples.

Aflatoxin B_1 (μM)	Iodine (mM)					
	0	1.3	0.65	0.26	0.13	0.026
1.0	14	100	100	100	100	100
0.5	9	100	100	100	100	100
0.1	0	37	80	97	90	43
0.05	n.t.[a]	23	49	75	70	29
0.01	n.t.	4	6	16	13	5
0.005	n.t.	2	4	7	7	0

[a] n.t. = not tested.

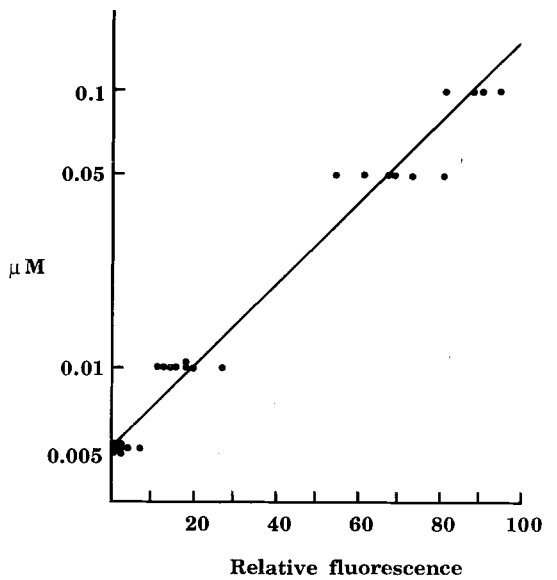

Fig. 2. Standard curve for aflatoxin B_1 (μM) in aqueous iodine determined by photofluorometry.

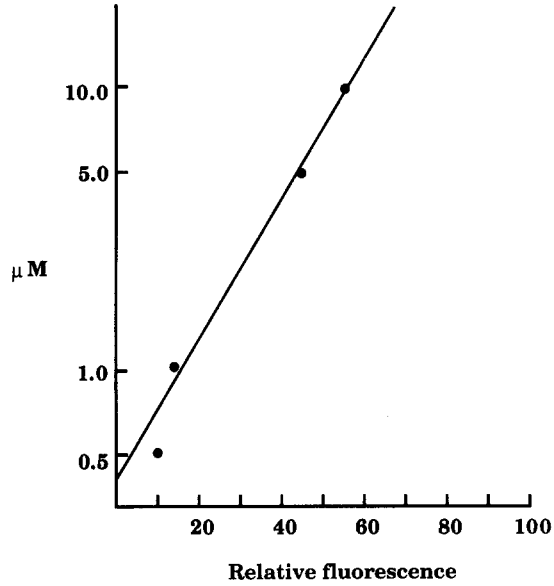

Fig. 3. Standard curve for aflatoxin B_1 (μM) in water determined by photofluorometry.

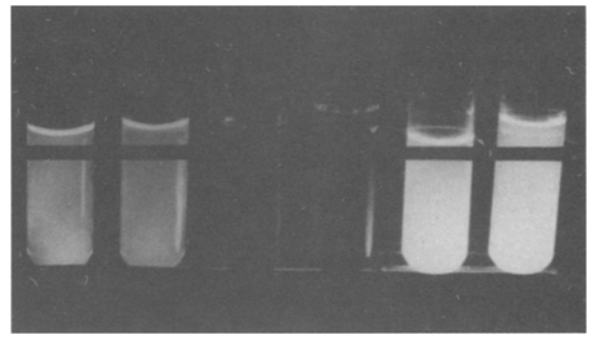

Fig. 4. Visual or qualitative comparison of strains Nos. 1, 2, and 3 for aflatoxin fluorescence of samples taken from 5-day CEB minicultures. Cuvettes contain, respectively in pairs from left to right, samples of Nos. 1, 2, and 3 diluted 100-fold in 0.26 mM aqueous iodine. Samples were examined and photographed under long-wave UV light.

tion in CEB. For this purpose, toxigenic strain No. 1 of *A. flavus*, nontoxigenic strain No. 2 of *A. flavus*, and toxigenic strain No. 3 of *A. parasiticus* were selected. Their evaluation was conducted in both 10-ml minicultures (Fig. 4) and in 100-ml cultures. Samples shown in Fig. 4 were analyzed by both HPLC and PFM (Table 2). The data in Table 2 compare strains Nos. 1 and 3 and indicate a substantial difference between the two strains for potential to synthesize aflatoxin in CEB after 3- and 5-day periods of growth.

The data in Table 3 compare these same two strains over a 10-day period and the resulting variation in time-course of aflatoxin synthesis from inoculation of 10 ml of CEB with varying levels of inoculum from 10^3 to 10^7 spores. In these latter experiments, the two strains proved to be rather comparable for aflatoxin yield, with both strains attaining highest titer after 10 days from an inoculum of 10^5 spores. A major difference between these two sets of experiments was that the spores used in

Table 2

Comparative PFM and HPLC analyses of aflatoxin production in CEB minicultures

The analyses were performed by relating PFM values and HPLC fluorescence over gain (fl/gn) units to ng/ml of aflatoxin B_1 from standard curves, respectively shown in Figs. 1 and 2A, and then multiplying by the reciprocal of a dilution factor (rdf).

Fungal strain	Day 3						Day 5					
	PFM			HPLC			PFM			HPLC		
	value	rdf	ng/ml	fl/gn	rdf	ng/ml	value	rdf	ng/ml	fl/gn	rdf	ng/ml
1	80	10^2	3500	0.44	10^4	3300	40	10^3	6800	0.64	10^4	3600
3	75	10^3	23500	1.0	10^3	46000	30	10^4	47500	1.13	10^3	50000

Table 3

PFM analysis of aflatoxin production in CEB minicultures with increased levels of inoculum

The analysis was performed by relating PFM values to ng/ml of aflatoxin B_1 from the standard curve shown in Fig. 2A and then multiplying by the reciprocal of a dilution factor (rdf).

No. of spores	Day 3			Day 7			Day 10		
	value	rdf	ng/ml	value	rdf	ng/ml	value	rdf	ng/ml
Strain No. 1									
10^3	30	10^2	430	45	10^3	7000	17	10^3	2800
10^5	3	10^3	1800	40	10^3	6000	56	10^3	10000
10^7	2	10^3	1600	7.5	10^3	2000	5.5	10^3	1900
Strain No. 3									
10^3	4	10^2	180	12.5	10^3	2400	34	10^3	4800
10^5	30	10^2	430	27	10^3	4200	45	10^3	7000
10^7	3	10^3	1800	26	10^3	3800	44	10^3	6900

Table 2 experiments were freshly harvested, whereas the spores used in the experiments of Table 3 and in subsequent experiments were either stored at 4°C for up to 30 days (strain No. 1) or were obtained from a subculture of the initial spore suspension of strain No. 3. The initial spore suspensions, the stored spore suspension of strain No. 1, and the spore suspension from a subculture of strain No. 3 were each adjusted to a viable spore titer of 10^9 spores per ml.

Although strain No. 2 was also grown in CEB, no aflatoxin was detected for this strain by PFM nor by HPLC in 3-, 5-, and 7-day old cultures. HPLC analysis of 10-day-old cultures of strain No. 2 revealed a broad and shallow peak suggesting the presence of as much as 200 ng/ml of aflatoxin. This result was not confirmed, however, by TLC nor by PFM analyses and most likely represents the formation of some fluorescent metabolite other than aflatoxin in older CEB cultures by this strain.

Strains Nos. 1 and 3 were also evaluated by PFM and TLC, after 100 ml of CEB were inoculated with 5×10^7 spores and incubated for 7 and 10 days. Quantitative data are presented in Table 4 and indicate good correlation between PFM and TLC analyses.

Finally, TLC analysis of the mixed standard containing aflatoxins B_1, B_2, G_1, and G_2, dissolved in 0.13 mM of aqueous iodine, indicated detention of the migration of aflatoxin B_1 and G_1, the major

Table 4

Comparative PFM and TLC analysis of aflatoxin production in CEB flask cultures

TLC was conducted on noniodinated and chloroform-extracted aflatoxins and quantitated by comparisons with known amounts of aflatoxins B_1, B_2, G_1, and G_2 in a mixed standard.

Fungal strain	Day 7				Day 10			
	PFM			TLC	PFM			TLC
	value	rdf	ng/ml	ng/ml	value	rdf	ng/ml	ng/ml
1	36	10^3	5200	6250	56	10^3	11000	9500
3	42	10^3	6300	6250	30	10^3	4500	3100

components of the standard. Fluorescence in this sample remained at or near the origin on the TLC plate and was not resolvable into components. HPLC analysis had demonstrated previously that aflatoxins B_1 and G_1 are preferentially enhanced for fluorescence by iodine and can be resolved by slightly different retention times [5]. TLC analysis of the aflatoxin B_1 standard, added to CEB and diluted into 0.26 mM of aqueous iodine, revealed incomplete iodination of the aflatoxin B_1 in a 10 μM standard solution, whereas complete iodination occurred in the 1 μM and 0.1 μM standard solutions.

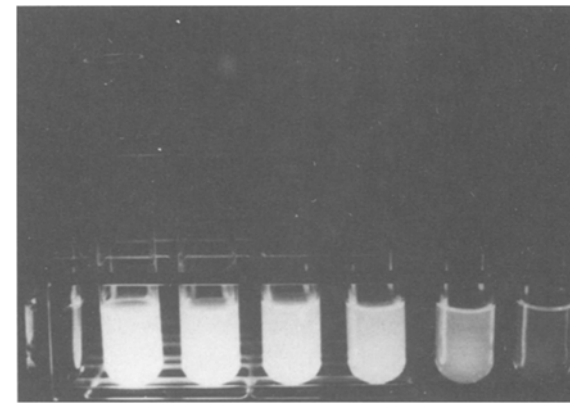

Fig. 5. Semiquantitative assessment of fluorescence for an aflatoxin B_1 standard examined under UV light. Cuvettes contain, respectively from left to right, 0.26 mM aqueous iodine, and 10 μM, 1 μM, 0.5 μM, 0.1 μM, 0.05 μM, and 0.01 μM aflatoxin in 0.26 mM aqueous iodine.

DISCUSSION

The fluorescence of aflatoxins exposed to long-wave (365 nm) ultraviolet (UV) light has been useful in the development of analytical procedures for these compounds [6]. TLC and HPLC coupled with fluorescence detection of aflatoxin have been useful in this regard. These procedures generally involve the analysis of aflatoxin-contaminated laboratory and field samples after solvent extraction and provide quantitative assessment for aflatoxin in the range of 0.01–0.1 μM [1]. More recent methods involve immunodetection of aflatoxin [2], but these procedures, although sensitive, are generally applied to solvent-extracted aflatoxin and are relatively expensive and time-consuming.

The overall blue fluorescence of aflatoxin B_1 under UV light is apparent in aqueous solution, and that fluorescence is enhanced significantly by treatment with iodine [3,5]. Optimization and direct quantitation of iodine-enhanced aflatoxin both in aqueous solution and in a culture medium have been the subjects of this research.

Standard curves for iodine-enhanced aflatoxin B_1 in aqueous solution as well as in CEB indicate sensitivity for detection of aflatoxin of the order of 0.01 μM. Aliquots from CEB fermentations of *A. flavus* and *A. parasiticus* strains, serially diluted in aqueous iodine, have been analyzed directly by PFM and HPLC and compared quantitatively with standard curves. Even without analytical quantitation by PFM or HPLC, samples can be judged visually for fluorescent intensity under long-wave UV, since fluorescent intensity comparable to 0.01 μM iodine-enhanced aflatoxin is visible and incremental increases in fluorescent intensity are discernible at levels up to 10 μM (Fig. 5). Direct observation of samples under UV light, with visual comparison to spiked samples, provides a rapid and convenient short-cut, allowing inexpensive scale-up of the method for large screening programs. The CEB is turbid and not assayable by PFM or HPLC without either 100-fold dilution and/or filtration. However, visual assessment of aflatoxin in CEB is both rapid and semiquantitative. Both HPLC and TLC, although more time-consuming, are quantitatively more accurate at low aflatoxin levels and afford the advantage of physically separating component aflatoxins and distinguishing any fluorescence by interfering compounds in a given medium. Iodine-enhanced fluorescence of compounds other than aflatoxin in the uninoculated CEB was ruled out by both HPLC and TLC analyses, since the only fluorescence-enhanced derivatives, following treatment of samples, migrated consistently with iodinated standards of aflatoxin B_1 and G_1. However, substances in CEB medium other than aflatoxin apparently complex iodine and at high levels of aflatoxin in CEB, these substances compete for

iodine at the 0.26 mM level. This was indicated by an unexpected increase in fluorescence of aflatoxin following dilution from 10-fold to 100-fold into a constant amount of iodine, a phenomenon that might also be explained in part by increased dissolution of aflatoxin upon dilution from the CEB.

Spores derived from a subculture of strain No. 3 indicated a significant reduction in potential to produce aflatoxin (Tables 3 and 4) in comparison to yields obtained and reported in Table 2. The difference is unexplained, but this result points out the merit of using the PFM assay in determining the stability or monitoring the loss of stability for aflatoxin production by a specific strain of *A. flavus* or *A. parasiticus*.

Direct PFM analysis of iodine-enhanced aflatoxin in CEB provides investigators with increased potential to examine several physiological and genetic parameters affecting aflatoxin biosynthesis. It is now possible to examine fermentation properties of numerous cultures over a period of days. The method provides a semiquantitative assay for aflatoxin in fermentation cultures, allowing for genetic selection and scale-up for critical examination of toxigenic potential of *A. flavus* and *A. parasiticus* strains grown in a coconut-based medium. While this method has been adopted only to assay aflatoxin levels from growing cultures in the laboratory, it has potential as well to be developed as a substitute for direct assay of aflatoxin from contaminated field samples or other substrates.

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