Detection and estimation of aflatoxins using both chemical and biological techniques

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Abstract

Production of aflatoxins on both natural (rice and corn) and semisynthetic (YES) media was conducted using an identified toxin - producing strain of *Aspergillus flavus*. The *A flavus* strain was able to produce 4 types of aflatoxins, namely B_1, B_2, G_1, and G_2 on rice, corn, and YES media. Quantitative data showed that the concentrations of aflatoxins B_1 and G_1 produced were 52, 40.3, and 39.6; and 64.7, 45.0, and 58.0 μg for 50 g of rice, corn, and YES media, respectively. In comparison, the yielded amounts of aflatoxins B_2 and G_2 were much lower: 11.5, 17.9, and 17.5; and 28.9, 40.3, and 39.5 μg for 50 g of rice, corn, and YES media, respectively.

A bioassay was conducted using the following 5 standard bacterial strains: *Bacillus megaterium, Bacillus subtilis, Streptococcus faecalis, Staphylococcus epidermidis,* and *Paracoccus denitrificans* as well as a field strain of *Candida albicans.* All strains except *P denitrificans* showed varied degrees of inhibition when applied with crude aflatoxins at 5 to 40 μg/mL. The minimum concentration of crude aflatoxins needed to inhibit *P denitrificans* was 10 μg/mL. Moreover, *Candida albicans* was not inhibited at any concentration of aflatoxins applied in this work.

Both undiluted and diluted (1/10, 1/100, and 1/1000) bacterial broth cultures showed a direct relationship between the diameter of inhibition zones and the concentrations of crude aflatoxins. Mean diameters of (7.0 – 20.5), (5 – 14), (4.5 – 13.0), (3.0 – 12.0), and (1.5 – 11.0) mm were observed when various concentrations of aflatoxins were applied using *B megaterium, S epidermidis, S faecalis, B subtilis,* and *P denitrificans,* respectively.

Field trials were applied to testify the validity of our data. A 1/100 dilution was prepared from each strain of 4 different species to estimate aflatoxins in samples of contaminated corn. Both chemical and biological assays were carried out at the same time. Data revealed that the most sensitive organism inhibited by as low as 7.5 μg aflatoxins/mL was *B megaterium* giving an inhibition zone of 10.5 mm, followed by *S epidermidis* with an inhibition zone of 7.5 mm. In relation, the other 2 organisms were less sensitive to crude aflatoxins. Similarly, the biological assay was applied to detect aflatoxins in some samples of wheat, corn, peanut, rice, and poultry rations. Of the 14 wheat and 10 corn samples, only 4 wheat and 2 corn samples were found to be positive. The same results were obtained using TLC analysis.

Introduction

Among mycotoxins, aflatoxins are of great interest for researchers because of their carcinogenic nature for both humans and animals. In addition, aflatoxins occur in high frequency under natural conditions and may cause serious economic loss resulting from contaminated foods and feedstuffs. Losses are difficult to estimate in animals and birds, because they come about in many ways, mainly as a result of high mortality rate, low productivity, lower feed conversion rates as well as the losses due to the cost of controlling of fungal growth during storage of foods and feeds.

The detection and estimation of aflatoxins have been mainly carried out using official analytical techniques. Such techniques need either Thin Layer Chromatography (TLC) or High Performance Liquid Chromatography (HPLC). However, these methods are expensive and sometimes require using very expensive equipment such as HPLC. This fact has encouraged some investigators to try biological methods to detect and determine aflatoxins in different materials. Generally, biological methods are much cheaper than chemical ones.

The present work was planned to investigate the antibacterial activity of crude aflatoxins on different standard bacteria as well as on *Candida albicans,* with the aim of adopting a microbial inhibition test to detect and possibly estimate aflatoxins in foods and feeds.

Materials and methods

Production of aflatoxins was conducted using a local *A flavus* isolate from the Dept of Microbiology, Fac of Vet Med, Cairo University. After the identification of the isolate as recommended by Ajello et al (1), the prepared spore suspension of the inoculum was added to the natural substrate (rice and corn) as described by Shotwell et al (2) and to a (2% and 20% w/w) yeast extract sucrose (YES) broth and subsequently incubated at 25°C for 8 days as described by Davis et al (3). Extraction of crude aflatoxins was carried out according to the minicolumn detection method of AOAC (4). The obtained extracts were divided into two unequal parts, the smaller one was examined chemically for quantitation of aflatoxins B_1, B_2, G_1, and G_2, while the second part was kept for bioassay.

Chemical analysis

Standard solutions of aflatoxins B_1, B_2, G_1, and G_2 were prepared by dissolving each of the crystals (obtained from Sigma Chemical Co, USA) separately in a benzene - acetonitril mixture (98 : 2, v/v) and then checked according to the AOAC method (1980). Separation and qualitation had been carried out by TLC. Aflatoxins B_1, B_2, G_1, and G_2 were detected visually using a UV lamp with long (366) and short (254) wavelengths. The quantity of aflatoxins in the extracts was determined by comparing the densitometer fluorescence reading of standard spots with the intensity of sample spots in the linear correlation of standard curve.

Biological assay

Standard strains of *B megaterium* (a sporogenous strain Km), *B subtilis* (strain 60015), and *P denitrificans* (gram negative soil strain with an electron transport chain) were obtained from the Michigan State University Dept of Microbiology and Public Health. Strain (No 12228) of *S epidermidis* and (No 19433) of *S faecalis*

came from the American Type Culture Collection (ATCC), Maryland, USA. All these standard isolates as well as a field yeast strain of *Candida albicans* were used to screen the microbial effect of crude aflatoxins. The concentrations of 40, 30, 20, 15, 10, and 5 μg crude aflatoxins per mL (5 % acetone in water) were applied using the Gutter technique (5).

Field application

50 samples of peanuts, corn, rice, wheat, and poultry rations were collected randomly from the markets and examined chemically for aflatoxins using minicolumn detection method (4). Subsequently they were biologically examined to testify the validity of this bioassay.

Results and discussion

Table 1 shows the quantity of aflatoxins produced by *A flavus*. The appropriate *A flavus* strain was capable of producing 4 types of aflatoxins, namely B_1, B_2, G_1, and G_2. The highest concentration of yielded aflatoxin was of G_1, while the lowest concentration obtained was of aflatoxin B_2.

Shotwell et al (2) also obtained the 4 types of naturally occurring aflatoxins B_1, B_2, G_1, and G_2 using standard strain of *A flavus* (NRRL, 2999) when grown on rice. Their aflatoxin values were higher compared to our corresponding values. This might be mainly due to differences in the *A flavus* strain used as well as the incubation temperature time and aeration (6, 7).

Regarding each aflatoxin, the concentration of aflatoxin G_1 was higher than all other aflatoxins on rice, corn, and YES broth (Table 1). Shotwell et al (2) recorded earlier that the highest amounts yielded on rice were of aflatoxin B_1. This difference might be caused by the incubation temperature or the particular fungus strain as mentioned by Rabie and Smalley (8). They reported that the most important factor controlling the proportion of aflatoxins B_1 to G_1 produced by *A flavus* was temperature. They added that the optimal temperature to produce aflatoxin B_1 was 24°C, while the optimum for G_1 was 30°C.

Regarding antibacterial activity of the crude aflatoxins, data showed that *B megaterium, S epidermidis, S faecalis,* and *B subtilis* were widely inhibited by aflatoxin concentrations ranging from 5 – 40 μg/mL (obtained from rice and calculated as the sum of the 4 aflatoxin concentrations found by TLC). However, *P denitrificans* was not inhibited by a concentration of 5 μg/mL and inhibition occurred only with concentrations of 10 μg/mL crude aflatoxins or more (Table 2). On the other hand, *Candida albicans* was not inhibited at any concentration used.

Tab 1: Quantity of aflatoxins produced on natural substrates (rice and corn) and yeast extract sucrose (YES) broth

Substrate/broth		Concentrations of aflatoxins (μg/50 g grains or 100 mL broth)			
		B_1	B_2	G_1	G_2
Rice	(50 g)	52.0	11.5	64.7	28.9
Corn	(50 g)	40.3	17.9	45.0	40.3
(YES) broth	(100 mL)	39.6	17.5	58.9	39.5

Tab 2: Mean values of inhibition zones induced by crude aflatoxins

Density of bacterial suspension (count/mL)	Aflatoxin concentrations/mL				
	5 μg	10 μg	20 μg	30 μg	40 μg
	Bacillus megaterium (Sporogenus strain KM)				
5.0 x 10^4	7.0	7.5	11.0	12.0	14.0
5.0 x 10^3	10.0	11.0	12.0	14.0	15.5
5.0 x 10^2	10.0	11.5	14.5	16.0	17.0
5.0 x 10^1	14.0	15.0	16.0	20.0	20.5
	Staphylococcus epidermidis (ATCC, No 12228)				
4.8 x 10^4	5.0	6.5	8.0	9.5	12.0
4.8 x 10^3	5.5	7.0	8.5	9.5	13.0
4.8 x 10^2	7.0	7.5	8.5	11.0	14.0
4.8 x 10^1	8.0	10.0	10.5	11.5	14.5
	Streptococcus faecalis (ATCC, No 19433)				
4.2 x 10^4	4.5	6.0	7.5	8.5	11.0
4.2 x 10^3	4.5	6.5	7.5	9.0	12.0
4.2 x 10^2	6.0	7.0	8.0	9.5	12.5
4.2 x 10^1	7.0	8.2	9.0	10.5	13.0
	Bacillus subtilis (Strain 60015)				
4.5 x 10^4	3.0	4.0	5.0	6.0	8.0
4.5 x 10^3	4.0	5.5	7.0	7.5	10.0
4.5 x 10^2	5.0	6.0	7.0	8.0	10.5
4.5 x 10^1	6.5	8.0	8.5	10.5	12.0
	Paracoccus denitrificans				
3.2 x 10^4	–	1.5	2.0	2.5	4.0
3.2 x 10^3	–	3.0	4.5	5.5	8.0
3.2 x 10^2	–	5.0	5.5	7.0	9.0
3.2 x 10^1	–	6.5	7.5	9.5	11.5

Many scientists have studied the antibacterial activity of aflatoxins on *B megaterium* (9 – 12). They demonstrated that concentrations of 5 – 30 μg aflatoxins/mL had an inhibiting effect on *B megaterium*. Such wide divergence might be due to the *B megaterium* strains as the ratios between the 4 types of aflatoxins as reported by Wogan (13) who demonstrated that the potency of aflatoxin B_1 is roughly double and 4 - folds to that of aflatoxin B_2 and G_2, respectively. Table 2 shows clearly that the diameter of inhibition zone is not only dependent on the aflatoxin concentrations, but is also clearly influenced by the density of bacterial suspension used for the test (count/mL). This indicates the need for standardization of such bacterial suspensions. These findings are in accordance with those obtained by Buckelew et al (11) and Tiwari et al (12) who found that the effect of aflatoxins on *B megaterium* cells depended on their concentration with a linear relationship in the death curve. They added that bacteriostatic action of aflatoxin B_1 was observed at lower concentrations (8 – 16 μg/mL), while a higher concentration (32 μg/mL) caused an irreversible bactericidal action on *B megaterium*.

Similarly, *S epidermidis, S faecalis*, and *B subtilis* showed inhibition zones starting from 3 – 8 mm to 8 – 14.5 mm corresponding to crude aflatoxins concentrations of 5 and 40 μg/mL, respectively. This data varies from previous findings (9) re-

Tab 3: Mean values of inhibition zones (mm) obtained from the 6 (ve⁺) samples of contaminated wheat and corn

Standard bacterial strains	Sample 1	Wheat Sample 2	Inhibition zones (mm) Sample 3	Sample 4	Corn Sample 5	Sample 6
Bacillus megaterium	12.0	12.0	10.5	11.5	13.0	10.0
Staphylococcus epidermidis (ATCC No 12228)	8.0	8.0	7.2	7.5	7.0	6.0
Streptococcus faecalis (ATCC No 19433)	7.5	7.5	6.5	7.0	6.0	5.5
Bacillus subtilis (60015)	6.5	6.5	5.5	6.0	5.0	4.5

* The densities of the tested 4 bacterial strains were 5×10^2, 4.8×10^2, 4.2×10^2, and 4.5×10^2 per mL, respectively.

cording that members of the Microccaceae family (including Staph) were not inhibited by as high as 30 µg/mL of crude aflatoxins.

P denitrificans showed no inhibition at a concentration of 5 µg/mL in any of the bacterial dilutions used, but was inhibited at 10 µg/mL and higher concentrations up to 40 µg/mL. It has previously been used as a model organism to assay the toxicity of aflatoxins because its electron transport chain is similar to that of the mammalian cell mitochondria (5). In general, the inhibition zones are the network of bacterial species, strain, density of bacterial suspension (count/mL), and quantity of each aflatoxin used. Therefore these findings indicate the need to standardize the strain and the density of bacterial suspension in order to get reproducible and comparable results.

The *Candida albicans* isolate was resistant to the highest concentration of crude aflatoxins tested (40 µg/mL). This result is parallel to that of Burmeister and Hesseltine (9) who found that 38 species of yeasts from 6 different genera were not inhibited by 30 µg/mL of aflatoxins.

The biological assay to detect aflatoxins in some field commodities showed that 6 out of 50 samples were positive (using both the biological and TLC assays) while the remaining samples were negative in both tests. However, it should be noted that the biological test is only a screening test whose result should be confirmed by TLC, firstly in order to exclude the inhibition of bacteria due to other chemicals or any biologically active materials and secondly to determine the type and exact amounts of aflatoxins.

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