Separation and Purification of Aflatoxins B_1, B_2, G_1 and G_2, and Comparison of Semi-Synthetic Aflatoxins B_2 and G_2 With Naturally-Occurring Aflatoxins B_2 and G_2

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Abstract

A method is described for the isolation of highly purified aflatoxins B_1, B_2, G_1 and G_2 from extracts of *Aspergillus flavus*. The four aflatoxins, isolated from background impurities by rapid passage of the extracts through an acid alumina column, are separated from each other by chromatography on a silica gel column. Aflatoxins B_2 and G_2 are prepared by hydrogenation of the mixture of aflatoxins B_1, B_2, G_1 and G_2 and then separated by elution from a silica gel column with chloroform containing 0.7% ethanol. A comparison of semi-synthetic aflatoxins B_2 and G_2 with naturally-occurring aflatoxins B_2 and G_2 shows no significant difference in physical properties.

Introduction

The aflatoxins are a group of toxic compounds elaborated by the mold *Aspergillus flavus*. The aflatoxins can be extracted from *A. flavus* cultures with chloroform, and a crude precipitate of the aflatoxins can be obtained by adding hexane to the chloroform extract. The precipitates usually contain about 50% total aflatoxins. None of the reported column chromatography procedures completely separate the toxins, although a liquid-partition chromatographic procedure developed by Robertson et al. (1) comes very close to accomplishing this objective. Isolation of the aflatoxins from the crude *A. flavus* extract and subsequent separation of the four aflatoxins, B_1, B_2, G_1 and G_2, whose structures are shown in Figure 1 (2) are difficult because of three factors: the presence of many interfering components in the *A. flavus* culture extracts; the close similarity of the chemical structures of the four compounds; and the great relative difference in the amounts of the four toxins produced by the mold. The amounts of aflatoxins B_2 and G_2 produced by the strains of *A. flavus* we have been using are usually only about 1% of the total amount of aflatoxins produced. The second and third factors cannot be eliminated but the first should be readily resolved.

Initial work on the separation of the four aflatoxins was directed toward eliminating interfering compounds from crude culture extracts. Successful removal of the interfering substances was followed by column chromatography of the purified aflatoxin mixture to separate the four aflatoxins. Because of the small amounts of aflatoxins B_2 and G_2 produced by the molds, they are usually prepared by catalytic hydrogenation of aflatoxins B_1 and G_1 (1,3,4). Hydrogenation of the purified aflatoxin mixture (B_1, B_2, G_1 and G_2) instead of the separated aflatoxins led to a mixture of B_2 and G_2 which was readily resolved.

Experimental Procedures

The removal of interfering materials from crude culture extracts was accomplished by rapid chromatography of crude extracts of *A. flavus* cultures, containing aflatoxins B_1, B_2, G_1 and G_2, through an acid alumina column with benzene-chloroform, 5:1, and benzene-chloroform, 1:1, as the eluting solvents. Two major fractions were obtained: one contained aflatoxins B_1, B_2 and G_1, and a trace of aflatoxin G_2; the second contained aflatoxin G_2 and a number of materials with low R_f values. Further work on the second fraction was non-productive; pure aflatoxin G_2 could not be obtained by additional column chromatography. The elution with benzene-chloroform, 5:1, gave a colorless solution of the four aflatoxins. However, it was necessary to collect this column eluate in a number of fractions, since a yellow compound was eluted as a discrete band within the larger aflatoxin band and had to be collected as a separate fraction. This fraction contained some aflatoxin, but was only a small part of the total aflatoxin-containing eluate. The colorless fractions, when combined and slowly evaporated to a small volume, yielded a colorless, crystalline material which was a mixture of the four aflatoxins. Only the four aflatoxins could be seen by TLC examination (fluorescence and iodine staining) of the crystalline product. The first of the three difficulties in

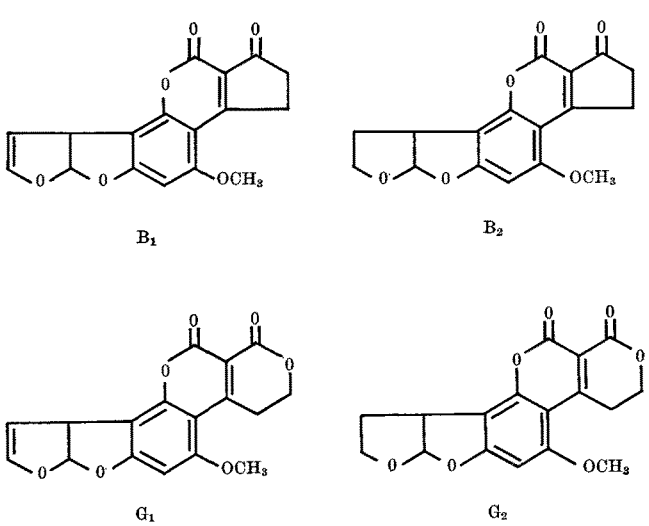

FIG. 1. The aflatoxins.

[1] Presented at the AOCS-AACC Meeting, Washington, D.C. April, 1968.

TABLE I

Ultraviolet Absorption of the Aflatoxins

Aflatoxin	MeOH λ max (mμ)	ε
B_1	223	21,700
	265	12,400
	361	21,900
B_2	222	20,100
	265	13,000
	362	24,600
G_1	216	28,100
	242	10,350
	264	10,400
	362	18,700
G_2	216	25,400
	244	10,900
	257	8,500
	265	9,200
	361	19,600

separating the aflatoxins was eliminated by this simple procedure, and chromatographic column separations of this well-defined mixture proved to be highly reproducible.

The crystalline mixture of the four aflatoxins was applied to a silica gel column and the mixture was resolved by elution with benzene, benzene-chloroform mixtures, chloroform, and chloroform-methanol, although rechromatography of the aflatoxin B_2-rich fraction on a second column was necessary to isolate pure aflatoxin B_2. Each of the four aflatoxins was recrystallized from hot chloroform until the UV absorptivities of the compounds were constant. The values of absorptivity for each of the aflatoxins are given in Table I.

It should be noted that crystalline aflatoxin develops a static charge and, if handled in the open, will become airborne and will contaminate the working area. To eliminate this hazard, it is necessary to carry out all operations with crystalline aflatoxin within the confines of a glove box (5).

The production of aflatoxins B_2 and G_2 in large amounts by catalytic hydrogenation of the crystalline mixture of aflatoxins B_1, B_2, G_1 and G_2 also led to the production of a small amount of the more fully reduced aflatoxin B_1, tetrahydrodesoxoaflatoxin B_1 a compound which had been prepared and characterized earlier (2). The aflatoxins B_2 and G_2 were easily separated from the tetrahydrodesoxo product, and also from each other, by chromatography on a silica gel column. The semi-synthetic toxins were recrystallized from hot chloroform.

Naturally-occurring aflatoxins B_2 and G_2 were compared with the semi-synthetic aflatoxins (Table II) with reference to ultraviolet spectra, mass spectra, TLC behavior, and fluorescence characteristics. In every case there was no significant difference between the naturally-occurring and the semi-synthetic aflatoxins B_2 and G_2. Preliminary studies of the toxicity to the chick embryo of naturally-occurring and semi-synthetic aflatoxins B_2 and G_2 reveal no significant differences; however, a future report will deal with the details of the comparative toxicology of these two pairs of aflatoxins.

Production and Extraction

Aspergillus flavus (FDA strain number M-93) was grown on 100 g sterilized shredded wheat and 60 ml of water in a 4 liter Fernbach flask for two weeks at room temperature. Twenty-five grams of anhydrous Na_2SO_4 were added to the flask after 700 ml of $CHCl_3$ was added. The mixture was boiled on a steam bath for 10 min and the $CHCl_3$ extract was decanted through a double thickness of Whatman No. 1 filter paper. The culture was extracted with two additional 500 ml portions of $CHCl_3$. The combined $CHCl_3$ extract was evaporated on a steam bath under a nitrogen stream to ca. 200 ml. Hexane was added dropwise until the solution became cloudy. The solution was allowed to stand at 0 C for 4 hr. The precipitate which formed was collected and air-dried in a glove box; the yield of crude extract was 210 mg.

Acid Alumina Column Chromatography of Crude Aflatoxin Extracts

The crude extract of the aflatoxins (1 g) was dissolved in the minimum amount of hot chloroform and applied to a 28 mm i.d. column prepared with 100 g Baker Reagent Acid Aluminium Oxide (for chromatography) in benzene. The column was eluted with benzene-chloroform (5:1 v/v) and ten 250 ml fractions were collected. The third fraction was highly colored; however, the other nine fractions were colorless. Aliquots from each of the 10 fractions were analyzed on TLC (6), and those fractions which showed only aflatoxins B_1, B_2, G_1 and G_2 (fractions 4–9) were combined and evaporated to ca. 75 ml. When allowed to stand overnight at room temperature

TABLE II

Comparison of Naturally-Occurring Aflatoxins B_2 and G_2 with Semi-Synthetic Aflatoxins B_2 and G_2

Criteria	Natural B_2	Synthetic B_2	Natural G_2	Synthetic G_2
Toxicity in Chick Embryo	Preliminary studies show *no* difference between natural and semi-synthetic materials.			
Fluorescence intensity as measured with densitometer	By means of regression analysis, it can be shown that the relationship of the amount of aflatoxin to the fluorophotometric response is the same whether the aflatoxin is natural or semi-synthetic.			
	MeOH λ max / ε	MeOH λ max / ε	MeOH λ max / ε	MeOH λ max / ε
UV	362 / 24,600	362 / 24,000	363 / 19,600	361 / 20,200
			265 / 9,200	265 / 9,800
	265 / 13,000	265 / 13,600	259 / 8,500	257 / 8,900
	222 / 20,100	222 / 21,000	243 / 10,900	244 / 12,000
d.p.[a]	302–302.5°	302–303.5°	250–254°	251–253°
TLC[b] CHCl₃/acetone, 9/1	$R_f = .62$	$R_f = .62$	$R_f = .43$	$R_f = .43$
BEW	$R_f = .59$	$R_f = .59$	$R_f = .41$	$R_f = .41$
	R_f values are averages from three plates run in each system.			
Mass Spectra	m/e = 314 (100%)	m/e = 314 (100%)	m/e = 330 (100%)	m/e = 330 (100%)
	m/e = 285 (21%)	m/e = 285 (21%)	m/e = 301 (12%)	m/e = 301 (8%)
	m/e = 271 (56%)	m/e = 271 (56%)	m/e = 287 (25%)	m/e = 287 (23%)
	m/e = 115 (15%)	m/e = 115 (16%)	m/e = 115 (14%)	m/e = 115 (14%)

[a] Mixture melting points are meaningless since all of these compounds decompose upon heating.
[b] Superimposed spots of B_2 (natural + synthetic and G_2 (natural + synthetic) each gave a single spot when developed with $CHCl_3$/acetone, 9/1.

the solution gave colorless crystals (420 mg) which were collected and air dried in a glove box. A TLC analysis (fluorescence and iodine staining) of solutions of the crystals showed that only aflatoxins B_1, B_2, G_1 and G_2 were present.

Continued elution of the acid alumina column with benzene-chloroform (1/1 v/v) affected the removal of the remaining amount of aflatoxin G_2 and a number of low R_f materials.

Silica Gel Column Chromatography of Aflatoxin Mixture

Two hundred milligrams of the purified aflatoxin mixture was applied to an 80 g silica gel column (Merck, 0.05–0.2 mm; column dimensions: 2.5 × 35 cm) which was prepared in benzene. The column was eluted at a flow rate of ca. 80 ml/hr with the following solvent series: benzene (0.5 liters); benzene/$CHCl_3$, (3/1, 2/1, 1/1, 1/2, 1/3 v/v, 1.5 liters each), $CHCl_3$ (1.5 liters); and $CHCl_3$/MeOH (98/2, 9/1 v/v, 1.5 liters each). The fraction size was ca. 13 ml. Every tenth fraction was analyzed by TLC chromatography. In Table III are presented the results of a typical silica gel column chromatograph. The weights of the toxins were obtained by combining fractions of the same content and evaporation of the combined fractions to dryness. The fraction which contained aflatoxin B_1 + B_2 (12 mg) was applied to a 6 g silica gel column (Merck, 0.05–0.2 mm) and the column was eluted with $CHCl_3$ which contained ca. 0.7% ethanol (normal preservative concentration). The eluate was collected in fractions of ca. 1.5–2 ml and after 70 fractions the chromatography was complete. Aliquots from every tenth fraction were spotted and analyzed on TLC plates; fractions 52–67 contained pure aflatoxin B_2 (4.5 mg). The same procedure was effective for the separation of aflatoxin B_2 from aflatoxin G_1. Separation of 22 mg of the B_2–G_1 mixture obtained from the first column (see Table III) gave 2.2 mg pure aflatoxin B_2.

Crystallization of the Aflatoxins

Each of the four aflatoxins was recrystallized by dissolving in hot chloroform and concentrating the solutions under a stream of nitrogen. The resulting saturated solutions were allowed to stand at 0 C overnight. The crystals were collected by suction filtration. Each toxin was recrystallized until the molar absorptivity of the sample was constant (B_1, 4 times; B_2, 3 times; G_1, 5 times; G_2, 3 times). The molar absorptivities of the four aflatoxins are given in Table I. Aflatoxin B_1 had mp 264–267 C (dec.); B_2 had mp 302–302.5 C (dec.); G_1 had mp 262–263.5 C (dec.); and G_2 had mp 250–254 C (dec.).

Solutions of each of the four aflatoxins were prepared at concentrations of 50 μg/ml in $CHCl_3$ and 100 ng of each of the toxins was spotted on TLC plates. The developed plates showed that each of the toxins was free of any trace of the other three toxins. The only other fluorescent spot on the TLC plate was a very faint spot at the origin.

TABLE III

Results of Silica Gel Column Chromatography of 200 mg of an Aflatoxin Mixture

Fraction	Weight (mg)	Type
1–350	85	B_1
351–440	12	$B_1 + B_2$
441–670	22	$B_2 + G_1$
671–950	77	G_1
951–1090	2	G_2

The purified aflatoxins were stored in vials under a nitrogen atmosphere.

Catalytic Hydrogenation of Aflatoxin Mixture

One hundred milligrams of the crystalline aflatoxin mixture was dissolved in ethyl acetate. To the solution 200 mg of 5% $Pd/CaCO_3$ catalyst was added. The mixture was hydrogenated at 1 atm and at room temperature for 45 min. The mixture was filtered and the catalyst was washed with $CHCl_3$. A TLC analysis of the filtrate showed that aflatoxins B_1 and G_1 had been completely converted to aflatoxins B_2 and G_2, respectively; however, a trace of a third product, tetrahydrodesoxoaflatoxin B_1, could be detected at an R_f much higher than that of aflatoxin B_2.

Silica Gel Column Chromatography of the Aflatoxin B_2-G_2 Mixture

The mixture of tetrahydrodesoxoaflatoxin B_1 and aflatoxins B_2 and G_2 prepared by catalytic hydrogenation (100 mg) was applied to a 30 g silica gel column (Merck, 0.05–0.2 mm) and the column was eluted with $CHCl_3$. Fractions 1–15 (20 ml each) contained tetrahydrodesoxoaflatoxin B_1, fractions 21–29 contained aflatoxin B_2, and fractions 34–39 contained aflatoxin G_2. The semi-synthetic aflatoxins B_2 and G_2 were recrystallized in the same manner as the naturally-occurring compounds. Semi-synthetic aflatoxin B_2 had mp 302–303.5 C (dec.) and semi-synthetic aflatoxin G_2 had mp 251–253 C (dec.). The semi-synthetic aflatoxins were identical in all respects with the naturally-occurring aflatoxins B_2 and G_2 (Table II).

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