An Improved Separation of Aflatoxins[1]

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Abstract

Crude aflatoxin from a chloroform extract of *Aspergillus flavus* cultures on rice was precipitated with Skelly Solve B and chromatographed on 100–200 mesh silica gel columns, using ethyl acetate as eluant. On this column there was no separation of aflatoxins from each other, but most of the brown, oily material was removed. The next step in the purification was chromatography on 100–200 mesh silica gel columns with chloroform and 5% methanol/chloroform as eluants. A large part of the B_1 was purified, but B_2, G_1 and G_2 did not separate, and M_1 had a brown oil that prevented crystallization. The M_1 was purified by chromatography on Sephadex LH-20 with chloroform; the brown material was retained while the M_1 passed through. The separation of aflatoxin B_2, G_1, and G_2 was achieved by column chromatography on Silica Gel H for TLC. In addition, aspertoxin was separated and identified. The purity and identity of the compounds were established by 100 MHz NMR.

Introduction

TO OBTAIN LARGE QUANTITIES of pure, crystalline aflatoxins for analytical standards and for chemical and pharmacological studies, it was necessary to develop a method of extracting mold cultures of *Aspergillus flavus* and isolating the toxins in useful quantities. Survey of the literature showed that although many methods for the separation and purification of aflatoxins have been reported, a method for the complete separation of aflatoxins in large quantities was still lacking (1–3). With this improved procedure we were able to isolate, in addition to aflatoxins B and G, aflatoxin M_1 and aspertoxin from the culture extracts. High resolution NMR spectra were obtained to identify and demonstrate the purity of each compound.

Extraction

Cultures of *A. flavus* strain No. 2999 on rice (4) weighing 4.5 kg were first defatted by extraction for 8 hr with 4.5 liters Skelly Solve B in a percolator type extractor. This extract was checked for aflatoxin content with thin-layer chromatography on silica gel-cellulose plates (5) developed in 5% methanol/chloroform, found to have none, and so disposed. The cultures were then extracted three times for 8 hr each with 4.5 liters of chloroform. The UV absorption peak at 360 mμ of each extract was measured on a Cary 15 spectrophotometer. Since the third extract contained less than 70 mg/liter aflatoxin, the extraction was discontinued. The combined chloroform extracts were evaporated on a rotary evaporator, the residue dissolved in approximately 100 ml of chloroform, and the aflatoxins precipitated by the addition of about one liter Skelly Solve B. The crude aflatoxin, 7.55 g, was collected and estimated by UV absorption to contain about 5.2 g of aflatoxin.

Chromatography

The crude aflatoxin was first chromatographed on 50 × 1,000-mm columns of silica gel (Mallinckrodt SilicAR, CC-7, 100–200 mesh) prepared with anhydrous ethyl acetate. The columns were loaded with approximately 2 g of aflatoxin per kilogram of silica gel, and eluted with ethyl acetate. Most of the brown, oily material present in the crude mixture was thus removed but the aflatoxins were not separated from each other.

Next, the crude aflatoxin was chromatographed on 50 × 1,000-mm columns of silica gel prepared with chloroform. The aflatoxins were loaded 1 g/kg of silica gel. They were eluted from the column with chloroform until the B_1 started coming out, and then the solvent was changed to 5% methanol/chloroform by gradient elution, and continued with 5% methanol/chloroform until the fractions containing M_1 were out. For all the columns, the fractions were monitored by TLC. This procedure thus far allowed large amounts of B_1 to be separated from the rest of the metabolites. From this column pure B_1, 3.41 g after crystallization from chloroform/methanol, and about 2 g of mixed B and G were obtained. Also eluted was 19 mg of pure G_2, but for the most part, B_2, G_1 and G_2 were not separated.

The M_1 fractions contained a yellow oil which prevented crystallization. The removal of this oil was not effected by further chromatography on silica gel. The purification of M_1 was accomplished by chromatography on columns of Sephadex LH-20 prepared with chloroform and eluted with chloroform. From this column the aflatoxin could be eluted while the interfering oil remained adsorbed at the top of the column. The use of methanol as eluant was attempted, but was not as effective. The aflatoxin M_1, 38 mg, was crystallized from chloroform/methanol.

The complete separation of aflatoxins B_1, B_2, G_1 and G_2 could not be accomplished by any of the previously mentioned columns. Although thin-layer chromatography separated these compounds, this technique was too tedious to obtain any quantity of material by stripping the plates. Excellent resolution was obtained on columns of Silica Gel H for TLC (E. Merck) prepared with chloroform and eluted as before with chloroform and chloroform/methanol. On these columns we obtained virtually complete resolution of the four aflatoxins B and G. On a 25 × 1,000-mm column of Silica Gel H, we obtained 6 mg B_1, 31 mg B_2, 98 mg G_1 and 1 mg G_2 from 200 mg of mixed aflatoxins from the larger column. After crystallization from chloroform/methanol, NMR spectra were run.

Several problems were encountered in using columns made from the TLC adsorbent. Great care had to be taken to insure good columns. Unlike the coarser silica gel, the slurry had to be made very thin so that air bubbles would not become entrapped. The slurry had to be poured all at once to eliminate discontinuities in the column, a major cause of unsatisfactory columns. This required a removable extension to provide the extra volume. The column had to be packed under pressure, since gravity flow,

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in addition to being slow, was not sufficient to compact the adsorbent.

Another problem in using these columns was the pressure drop across the column. Under gravity alone, the flow rate was so slow that internal column mixing became evident. An inexpensive solution to this problem was to use the pressure from a compressed nitrogen cylinder. For general use, though, this was unsatisfactory; frequent solvent changes were required, depending on the size of the solvent reservoir at the top, and gradient elutions could not be used. We found a continuous, non-pulsating pump such as the Cheminert Metering Pump (Chromatronix, Inc., Berkeley, California) to be excellent for the job.

The purity of the aflatoxins obtained from these columns was checked by the NMR spectra. An impurity noted in the spectrum of aflatoxin M_1 running slightly ahead of M_1 on Silica Gel H columns was separated and identified as aspertoxin (6–8).

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