Aflatoxins M_1 and M_2: Preparation and Purification[1]

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Abstract

A crude product containing aflatoxins M_1 and M_2, as well as large quantities of aflatoxins B_1 and B_2, obtained by fermentation of rice with *Aspergillus flavus* NRRL 3251 was chromatographed on a silicic acid column. Almost all the B_1 and B_2 were separated from M_1 and M_2. Aflatoxins M_1 and M_2 were eluted together with ethanol-chloroform (5:95 v/v). The combined M_1 and M_2 fraction was placed on a Merck silica gel (0.05–0.2 mm) column to be washed with hexane-chloroform (1:1 v/v) and chloroform to remove traces of B_1 and B_2 and eluted with ethanol-chloroform (1.5:98.5 v/v) to obtain aflatoxin M_1 and mixtures of M_1 and M_2. Rechromatography of M_1 on another silica gel column gave pure M_1 which was crystallized from acetonitrile. Aflatoxin M_2 was prepared by hydrogenation of M_1 and crystallized from acetonitrile.

Introduction

Allcroft and Carnaghan in 1963 (1) found a new toxin in milk of cows fed aflatoxin-containing peanut meal. Holzapfel et al. (2) first isolated and characterized the milk toxin as aflatoxins M_1 and M_2, compounds structurally related to aflatoxins B_1 and B_2. Since it was reported that M_1 was as toxic to ducklings as aflatoxin B_1 (2,3) and since important food commodities were implicated, a method for obtaining quantities of M_1 and M_2 was needed for standards, animal feeding tests, metabolism studies and detoxification investigations.

Several workers (2,4–6) have isolated small amounts of aflatoxin M from excretions of animals fed aflatoxin B_1 or from molded peanuts or rice. Hesseltine et al. (7) surveyed approximately 70 strains of the *Aspergillus flavus* group for production of aflatoxins B_1, G_1 and M_1. Although this study revealed that aflatoxin M was not produced in large quantities, one strain, NRRL 3251, did produce more aflatoxin M than others surveyed. This manuscript describes the production of aflatoxin M_1 and M_2 on rice, the isolation of M_1 by column chromatography and the preparation of M_2 from M_1 by hydrogenation.

Experimental Procedures

Fermentation

Rice (12 kg) was inoculated with spore suspensions of *A. flavus* NRRL 3251 in 2.8 liter Fernbach flasks (300 g/flask) and incubated for six days at 28 C on rotary shakers as previously described (7). This organism belongs to a new taxon of *A. flavus* maintained by the ARS Culture Collection at this Laboratory and is A. F. Schindler's (FDA) M 141 isolated from walnuts (7).

Isolation

Molded rice was steeped in chloroform in covered Fernbach flasks according to the method of Shotwell et al. (8). Combined chloroform extracts were concentrated in vacuo and divided into volumes (~115 ml) containing about 2–2.5 g total aflatoxin. Each portion was treated with anhydrous sodium sulfate to remove water and then with decolorizing carbon and copper carbonate to remove pigmented contaminants (9). Dried chloroform extracts were added to *n*-hexane (10 vol) to precipitate aflatoxins. Crude precipitates (2.5–3 g) were air-dried and assayed by thin layer chromatography (TLC) and densitometry.

Thin Layer Chromatography

Silica gel thin layer chromatoplates (20 × 20 cm) (Adsorbosil-1, Applied Science Laboratories Inc., State College, Pa.) were prepared as previously described (10). Five microliters of standard aflatoxin solution (2.5 μg each of B_1 and M_1, 1.0 μg M_2 and 0.5 μg B_2/ml) were spotted on the same plate as 5 μl of properly diluted samples and developed in either solvent 1 (2-propanol-water-acetone-chloroform, 1:1.5:12:88 v/v/v/v) or solvent 2 (2-propanol-acetone-chloroform, 5:10:85 v/v/v) (Stubblefield and Shannon, unpublished data). Solvent 1 was used for assays of samples containing B_1, B_2, M_1 and M_2; solvent 2, for assays of samples containing only M_1 or an M_1-M_2 mixture. Both solvent systems resolve M_1 and M_2 on TLC plates. Developed plates were scanned and quantities of aflatoxins determined by densitometry (11).

Column Chromatography

A crude precipitate (2.5–3 g containing 80% total aflatoxins B_1, B_2, M_1 and M_2) was chromatographed on silicic acid column (2.5 × 30 cm) by the method of Shotwell et al. (8). The column was developed with ethanol (abs.)-chloroform (washed) (1:99 v/v) to elute aflatoxins B_1 and B_2 and with ethanol-chloroform (5:95 v/v) to elute aflatoxins M_1 and M_2. The latter solvent was added when B_1 and B_2 were almost eluted from the column. Flow rates were adjusted to 30 ml/hr, and 10 ml fractions were collected and monitored for the presence of aflatoxins by TLC. Aflatoxin M_1 and M_2 fractions were pooled, concentrated in vacuo to 10 ml and assayed.

A concentrated solution from silicic acid columns containing 40–45 mg M_1 and M_2 was mixed with silica gel (3 g) (Merck, 0.05–0.2 mm, Brinkmann Instruments, Inc., Westbury, N.Y.), and the mixture was carefully dried under nitrogen. The silica gel mixture was slurried in hexane and added to the top of a silica gel column (1.5 × 25 cm) packed as slurry in chloroform (ACS). The column was developed with hexane (50 ml), hexane-chloroform (1:1 v/v) (100 ml), chloroform (ACS) (100 ml) and ethanol-chloroform (1.5:98.5 v/v) (about 500 ml). Flow rates were maintained at 12–18 ml/hr and 5 ml fractions were collected when fluorescent material started to elute. All fractions comprised of M_1 alone were combined, concentrated in vacuo to 10 ml and assayed; mixtures of M_1 and M_2 were treated similarly. More free M_1 is obtained by rechromatography of the combined M_1-M_2 mixture.

A concentrate containing 24 mg M_1 from the first silica gel column was rechromatographed by the same procedure. The column was prepared and developed as previously described with the substitution

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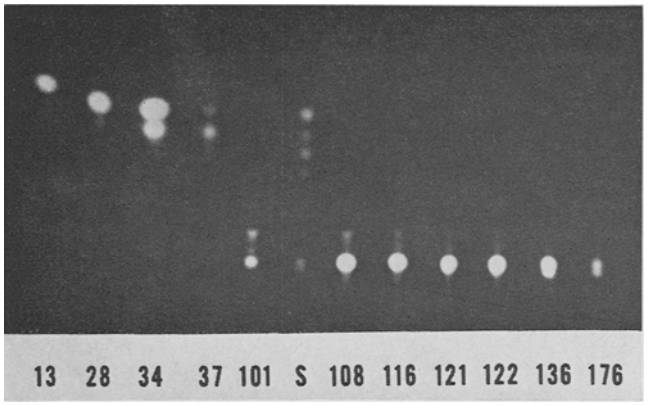

FIG. 1. Thin layer chromatogram of fractions eluted from first silica gel column with ethanol-chloroform (1.5:98.5 v/v) showing separation of aflatoxins B_1 and B_2 (13–37) and M_1 and M_2 (101–176). Thin layer plates coated with silica gel GA were developed in 2-propanol-water-acetone-chloroform (1:1.5:12:88 v/v/v/v). The letter S indicates control spots from a standard solution of B_1, B_2, G_1, G_2, M_1 and M_2 (top to bottom).

of ethanol-chloroform (1:99 v/v) for ethanol-chloroform (1.5:98.5 v/v). Fractions containing M_1 were combined, concentrated, assayed and dried under nitrogen.

Crystallization

Aflatoxin M_1 (20 mg, 98% pure by weight) was crystallized from acetonitrile. Pure crystalline aflatoxin M_1 (10 mg) had the following characteristics: λ_{max}^{MeOH} 226, 265 and 357 nm and mol wt 328 (mass spec.) (2). When M_1 was spotted and developed by TLC at a level of 500 ng, only one fluorescent zone was visible under UV light (366 nm).

Hydrogenation

Aflatoxin M_1 (15 mg) was hydrogenated (10 min, Pd/C) (2) in methanol-chloroform (15:85 v/v) to obtain aflatoxin M_2. Crystalline aflatoxin M_2 (5 mg, from acetonitrile) had the following characteristics: λ_{max}^{MeOH} 221, 265 and 357 nm and mol wt 330 (mass spec.) (2). When M_2 was spotted and developed by TLC at a level of 500 ng, only one fluorescent zone was visible under ultraviolet light (366 nm).

Results and Discussion

Yields of 20–25 mg M_1 and 2–3 mg M_2/kg substrate were obtained by rice fermentation with *A. flavus* NRRL 3251. Although these yields are not large when compared to yields of aflatoxin B_1 (1250 mg/kg), sufficient quantities of M_1 are produced for standards, microbiological tests and toxicological studies.

Since this *A. flavus* strain does not produce aflatoxins G_1 and G_2, M_1 and M_2 can be isolated from B_1 and B_2 by column chromatography more easily. At least 99% of the B_1 and B_2 present in the crude products is separated from M_1 and M_2 by chromatography on silicic acid columns. Aflatoxins M_1 and M_2 are not eluted with ethanol-chloroform (1:99 v/v). After almost all the B_1 and B_2 is eluted, ethanol-chloroform (5:95 v/v) is added to elute M_1 and M_2 together. Fractions containing M_1 and M_2 from silicic acid columns are highly colored and contain small amounts of B_1 and B_2. These contaminants are removed on silica gel columns. More than 90% of total toxin was recovered from these columns.

Combined M_1-M_2 fractions from silicic acid columns were not evaporated to dryness because the residues were difficult to redissolve. Solvents commonly used for aflatoxins, chloroform and methanol, did not dissolve aflatoxin M samples easily after most of the B_1 and B_2 had been removed. Therefore, any difficulty workers may have experienced in handling aflatoxin M might be due to solubility not instability. Aflatoxin M samples dissolve in methanol-chloroform (15:85 v/v) more readily than in either solvent alone.

Aflatoxin M in ethanol-chloroform from silicic acid columns could not be added directly to silica gel columns due to the large concentrations of ethanol present. Therefore, samples were mixed with silica gel, solvent was evaporated (under nitrogen) and each silica gel mixture was added in hexane to a column. Hexane-chloroform (1:1 v/v) was used to remove a green fluorescent band ahead of B_1 and B_2. Aflatoxins B_1 and B_2 were separated on columns from M_1 and M_2 with chloroform and eluted with ethanol-chloroform (1.5:98.5 v/v). When ethanol-chloroform (1.5:98.5 v/v) was added directly after hexane-chloroform (1:1 v/v), aflatoxins M_1 and M_2 eluted with B_1 and B_2. Most of the color contaminants remained on these columns.

A typical elution pattern of aflatoxins from the first silica gel columns was photographed from a TLC plate (Fig. 1). Aflatoxins B_1 and B_2 and other fluorescent impurities are eluted in fractions 13–37. Traces of these contaminants are present in fractions 101–121 which contain free M_1 (representing 60% of total recovered). Mixtures of M_1-M_2 were eluted in fractions 122–176. Recoveries of aflatoxins M_1 and M_2 from the silica gel columns were more than 90%. If methanol-chloroform (1.5:98.5 v/v) is substituted for ethanol-chloroform (1.5:98.5 v/v), aflatoxin M_1 is not separated from M_2. Although M_1 and M_2 are separated by rechromatography on silica gel columns with methanol-chloroform (1:99 v/v), recoveries of the toxins from these columns were only 70–80%.

Pure aflatoxin M_1 for crystallization was achieved by rechromatography on silica gel columns with ethanol-chloroform (1:99 v/v). Recoveries of M_1 were the same as those from the first silica gel columns. Aflatoxin M_1 was readily crystallized from acetonitrile.

Fermentation yields of aflatoxin M_2 are low; therefore, pure M_2 was not isolated by these methods. Hydrogenation of M_1 from a silica gel column produced pure M_2 as determined by TLC and viewed under ultraviolet light (366 nm).

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