Aflatoxins B_1, B_2, G_1, and G_2: Separation and Purification[1]

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Abstract

Aflatoxins B_1, B_2, G_1, and G_2 have been separated on a series of chromatographic columns. Chromatography of crude products isolated from molded wheat and rice on silicic acid with washed chloroform:ethanol (99:1) gave relatively pure B_1. The rest of the column fractions containing predominantly G_1, along with B_1, B_2, and G_2, were pooled and fractionated on a Silica Gel G column. The mobile phase was washed chloroform:acetone:ethanol (97.3:2.0:0.75). Thin-layer chromatography was used to follow column development. Each of the aflatoxins was treated with either decolorizing carbon or copper carbonate to remove colored pigments, and rechromatographed on Silica Gel G. Crystalline aflatoxins were prepared from chloroform solutions by addition of *n*-hexane, methanol, or ethanol.

Introduction

SINCE THE DEATHS of a large number of turkey poults in Great Britain in 1960 (1), a demand for a supply of the causative agents, aflatoxins, has arisen. These hepatotoxic metabolites are produced by certain strains of *Aspergillus flavus* and have been difficult to acquire. Originally, material for research on these compounds was obtained by isolating small quantities from preparative thin-layer plates (2,5). A sufficient supply was acquired for chemical characterization and initial toxicity studies, but animal feeding and degradation studies require larger quantities. Pure aflatoxins were needed for some of the experiments.

We have been studying production of larger amounts of aflatoxin on cereal grains and methods of separating the four aflatoxins by column chromatography. Recently, Robertson et al. (6) reported a method of separating moderate amounts of B_1 and G_1 by a liquid-partition column. Since very small quantities of B_2 and G_2 were separated, hydrogenation was used to produce these toxins. The production of aflatoxins on rice and wheat substrates has been reported by Shotwell et al. (7) and Stubblefield et al. (8). The present communication comprises a report of the separation and purification of large quantities of aflatoxins B_1 and G_1, along with smaller amounts of B_2 and G_2, from these substrates by chromatography on silicic acid and Silica Gel G columns.

Experimental Procedures and Discussion

The crude aflatoxin used was extracted from wheat fermented with NRRL 3145 and rice fermented with NRRL 2999. These cultures of *A. flavus* were supplied by the ARS Culture Collection at this Laboratory. Organism NRRL 3145 is the Commonwealth Mycological Institute's (CMI) 93080 (Austwick's strain V. 4065/4, isolated from South African peanuts) and organism NRRL 2999 is CMI 91019b (Austwick's strain V. 3734/10 from Uganda peanuts). Methods of fermentation and extraction have been previously described (7,8).

The following purification procedure was incorporated into the extraction method to remove many of the interfering pigments and other contaminants present in the crude extracts. After chloroform extracts were dried with anhydrous sodium sulfate, they were treated with about 10 g (8 mg/g substrate) of decolorizing carbon (Darco G-60, Atlas Chemical Co., Wilmington, Del.) for 5 min and filtered through diatomaceous earth (Hyflo Super Cel, Johns-Manville, New York). The filtrate was then treated with 15 g (12 mg/g substrate) of cupric carbonate (9) for 5 min, after which the aflatoxin was precipitated from a concentrated chloroform solution with *n*-hexane (10 volumes). All crude and purified products were assayed for homogeneity by thin-layer chromatography (TLC) and densitometry (8). Thin-layer plates (20 by 20 cm) were coated with either Silica Gel G-HR (7) (Brinkmann Instruments Inc., Westbury, N.Y.) or Adsorbosil-1 (Applied Science Labs, State College, Pa) to a 0.500-mm thickness. Plates were developed in acetone:chloroform (1:9 v/v) (ACS) (3) in an unlined unequilibrated tank. A typical fermentation on 2 kg wheat yields 1.5 g B_1, 150 mg B_2, 1.9 g G_1, and 250 mg G_2. Fermentations on 2 kg rice produce predominantly B_1 and yield 2.0 g B_1, 150 mg B_2, 300 mg G_1, and 40 mg G_2. Crude products usually contain 70–80% total aflatoxins by weight.

Chromatography of the crude products on silicic acid columns (analytical reagent, 100 mesh, Mallinckrodt Chemical, St. Louis, Mo.) by the method of Shotwell et al. (7) was used to separate a large portion of the aflatoxin B_1 (50%). Reproducible column results were achieved when the chloroform used in the solvent system was washed with water to remove the ethanol preservative and then dried with anhydrous sodium sulfate. Some of the colored pigments were also removed by this column.

The product containing B_1, B_2, G_1, and G_2 from the silicic acid treatment, 300 mg of total aflatoxins, was dissolved in washed chloroform and chromatographed on a column (2 by 30 cm) of Silica Gel G (10–40 μ, Brinkmann Instruments Inc.) using washed chloroform:acetone:ethanol (97.3:2.0:0.75 v/v) as a slurry and elution solvent. The flow rate was adjusted to 0.3 ml/min, and 5-ml fractions were collected and monitored for the presence of aflatoxins by the use of TLC. Photographs of the thin-layer plates are reproduced in Fig. 1. Aflatoxins are separated relatively free of one another. Pigments and fluorescing zones other than aflatoxin are removed later.

Fractions comprised of B_1, B_2, G_1, and G_2 and mixtures of B_2–G_1 and G_1–G_2 were pooled and assayed (Table I). The first pooled fraction has essentially 100% of the B_1 eluted from the column and very small amounts of fluorescing impurities. Fractions 86–90 are mainly composed of B_2. More than 70% of the aflatoxin B_2 recovered was collected before the G_1 started to elute. Fractions 95–145 contain G_1 (161 mg) and account for 80% of the total recovered from the column. Negligible amounts of B_2 in these fractions are easily removed on the final

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purification column. Finally, pooled Fractions 153–221 are mainly aflatoxin G_2. More than 95% of the aflatoxins placed on the column was recovered. Since 50% of the B_1 was separated by silicic acid column chromatography, larger amounts of aflatoxins B_2 and G_1 were isolated free of other toxins from the Silica Gel G column.

Aflatoxin B_1 products (450 mg, 90% pure by weight) collected from the silicic acid and Silica Gel G columns were combined and rechromatographed on another column of Silica Gel G, prepared as a slurry in washed chloroform:*n*-hexane (50:50 v/v). The column was washed with 50 ml of chloroform:*n*-hexane solvent prior to elution with washed chloroform:acetone:ethanol (97.3:2.0:0.75 v/v), as described previously. These changes permitted a better separation of the fluorescing contaminant (shown in the figure) and B_1.

Aflatoxin B_1 (342 mg, 99% pure by weight) was dissolved in chloroform (ACS) and treated with a small amount of decolorizing carbon for 5 min, filtered through diatomaceous earth, and the filtrate evaporated under nitrogen. Dried B_1 was dissolved in minimal chloroform (ACS) at 58C and crystallized by addition of ethanol (abs.). Crystalline B_1 was recrystallized with chloroform-methanol and, finally, chloroform-ethanol. The crystalline product (198 mg) was characterized as: mp 265.8–266.2C (decomp) (measured on a Mettler FP-1, Mettler Instrument Inc., Princeton, N.J.); λ_{max}^{EtOH} 225, 265, and 363 mμ (ϵ 27,300, 14,300, and 22,800, respectively). Found: C, 65.43; H, 4.08. Required: C, 65.22; H, 4.12. These values and those given for B_2, G_1, and G_2 below compare to other reported values (2,4,6,10). The absorption spectra data cited in the literature vary, especially for B_2 and G_2. These variations are an indication of the difficulty encountered in separating and purifying the aflatoxins.

Aflatoxin G_1 (413 mg, 90% pure by weight) from Silica Gel G columns was rechromatographed on the same columns which included a 0.5-cm layer of copper carbonate on top of the silica gel. Development and elution were as described. Copper carbonate removed most of the contaminating pigments present in the G_1 samples (9). A total of 344 mg (99% pure by weight) was recovered from two columns. Crystallizations were performed as described for B_1 except that copper carbonate was substituted for carbon. The crystalline G_1 (260 mg) had the following characteristics: mp 246.7–247.3C (decomp.) (Mettler FP-1); λ_{max}^{EtOH} 245, 265, and 365 mμ (ϵ 11,300, 11,000, and 18,600, respectively). Found: C, 62.34; H, 3.89. Required: C, 62.05; H, 3.92.

Aflatoxins B_2 (92 mg) and G_2 (83 mg) were collected from the columns until sufficient quantities of each were accumulated for crystallization. Each was rechromatographed on Silica Gel G columns to remove traces of other aflatoxins and impurities. Aflatoxin B_2 was crystallized three times from chloroform-hexane (ACS) following decolorization with carbon. The crystalline B_2 (24 mg) (mp 280–283C uncor.; decomp.) was chromatographically pure when spotted at a level of 1 μg and its absorption spectrum was as follows: λ_{max}^{MeOH} 265 and 363 mμ (ϵ 12,200 and 21,300, respectively). Aflatoxin G_2 was crystallized three times from chloroform-hexane (ACS) after treatment with copper carbonate. The crystalline G_2 (27 mg) (mp 229–231C uncor.; decomp.) was chromatographically pure when spotted at a level of 1 μg and it had an absorption spectrum as follows: λ_{max}^{MeOH} 244, 265, and 365 mμ (ϵ 16,100, 13,200, and 25,800, respectively).

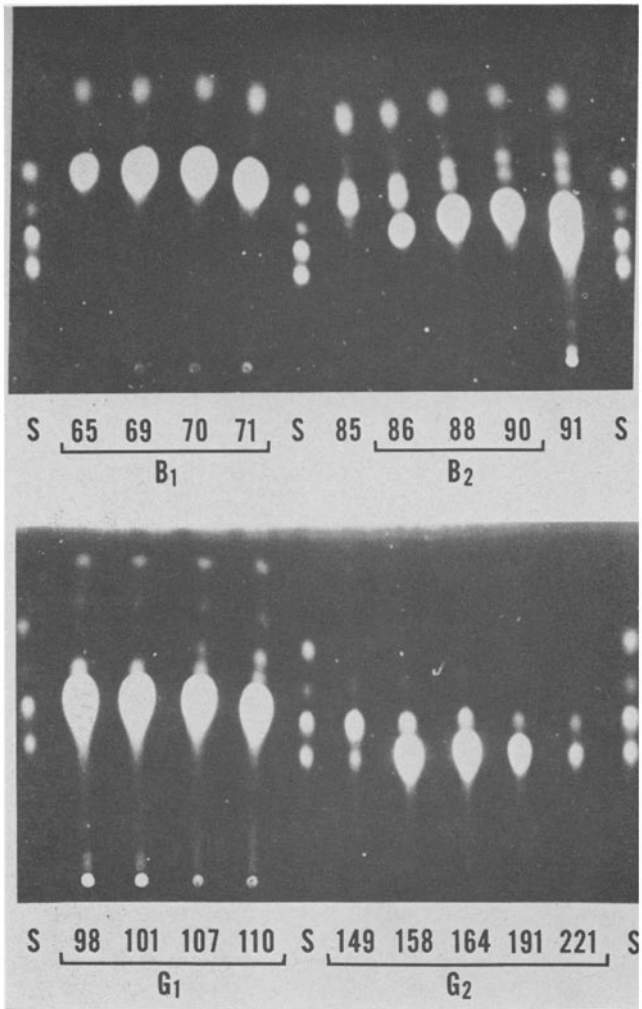

FIG. 1. Thin-layer chromatograms of fractions eluted on a Silica Gel G column with washed chloroform:acetone:ethanol (97.3:2.0:0.75) showing separation of aflatoxins B_1 and B_2 (top) and of G_1 and G_2 (bottom). Thin-layer plates coated with Silica Gel G-HR were developed with acetone:chloroform (1:9 v/v). The letter S indicates control spots from a standard solution of B_1, B_2, G_1, and G_2.

In summary, all four aflatoxins were separated

TABLE I

Separation of Aflatoxins B_1, B_2, G_1, and G_2 on Typical Silica Gel G Columns[a,b]

Pooled fractions	Aflatoxin content,[c] mg				Purity of isolated fractions,[d] %
	B_1(%)	B_2(%)	G_1(%)	G_2(%)	
65–85	35(100)[e]				84
86–90	Trace[f]	22(72)			75
91–94		8(28)	38(19)		94
95–145		Trace	161(80)		79
146–152			1(0.5)	1(3)	Discarded
153–221			Trace	25(97)	88
Totals	35	30	200	26	

[a] Developed and eluted with washed chloroform:acetone:ethanol (97.3:2.0:0.75 v/v).
[b] Recovery of total aflatoxin from column—over 95%.
[c] As determined by thin-layer chromatography and densitometry.
[d] Purity represented as per cent total aflatoxin by weight.
[e] Numbers in parentheses are percentages of total recovered from column.
[f] Represents less than 0.01 mg.

and isolated from crude precipitates obtained from molded wheat and rice. Silicic acid columns developed with washed chloroform:ethanol (99:1 v/v) separated 50% of the aflatoxin B_1 and other impurities. The remaining aflatoxins from this column were fractionated further into free B_1, B_2, G_1 and G_2, in good yields, on Silica Gel G columns developed with washed chloroform:acetone:ethanol (97.3:2.0:0.75 v/v). Individual aflatoxins were purified by chromatography on Silica Gel G for crystallization. Crystalline aflatoxins B_1, B_2, G_1, and G_2 have been prepared. Gram quantities of B_1 and lesser amounts of G_1 can be readily prepared by these procedures. Crystalline B_2 and G_2 are limited by the relatively small amounts produced in the fermentations.

REFERENCES

1. Allcroft, R., and R. A. B. Carnahan, Chem. Ind. (London), *1963*, 50–53.
2. Asao, T., G. Büchi, M. M. Abdel-Kader, S. B. Chang, E. L. Wick and G. N. Wogan, J. Amer. Chem. Soc. *87*, 882–886 (1965).
3. Eppley, R. M., J. Ass. Offic. Anal. Chem. *49*, 473–474 (1966).
4. Hartley, R. D., B. F. Nesbitt and J. O'Kelly, Nature *198*, 1056–1058 (1963).
5. Lijinsky, W., and W. H. Butler, Proc. Soc. Exp. Biol. Med. *123*, 151–154 (1966).
6. Robertson, J. A., W. A. Pons, Jr. and L. A. Goldblatt, J. Agr. Food Chem. *15*, 798–801 (1967).
7. Shotwell, O. L., C. W. Hesseltine, R. D. Stubblefield and W. G. Sorenson, Appl. Microbiol. *14*, 425–428 (1966).
8. Stubblefield, R. D., O. L. Shotwell, C. W. Hesseltine and M. L. Smith, Ibid. *15*, 186–190 (1967).
9. Wiseman, H. G., W. C. Jacobson and W. C. Harmeyer, J. Ass. Offic. Anal. Chem. *50*, 982–983 (1967).
10. Wogan, G. N., Bacteriol. Rev. *30*, 460–470 (1966).

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