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LARGE-SCALE PURIFICATION OF THE MYCOTOXINS AFLATOXIN B_1, B_2 AND G_1

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SUMMARY

The isolation and purification of gram quantities of the important mycotoxins aflatoxin B_1, B_2 and G_1 are described. The method involves final purification on a Waters Prep LC-500 instrument, loaded with silica cartridges, and elution with chloroform.

INTRODUCTION

The aflatoxins are the most important group of fungal toxins by reason of their frequent contamination of nutrition intended for human and animal consumption, and the extremely severe toxicological effects they exert on many mamalian systems on ingestion[1]. By virtue of this prominence they occupy a pivotal position in mycotoxin research and have been the subject of numerous studies on all aspects of their chemistry.

Chromatographic methodology has traditionally concentrated on the detection and analysis of these metabolites as contaminants of foodstuffs in increasingly smaller amounts, so that many reliable procedures exist for the determination of these compounds at the nanogram level[2]. These methods usually involve chromatography on reversed-phase silica gel which, because of its low capacity, is poorly suited to large-scale separations. However, comparatively little effort has been devoted to investigations at the other end of the scale, in which large quantities of the aflatoxins are produced and purified. Such methods as do exist generally use purification by preparative thin-layer chromatography (TLC) as a final step, because of the poor separation of aflatoxins B_1 (1), B_2 (2), G_1 (3) and G_2 (4) by conventional column chromatography[3]. That procedure is obviously cumbersome for large quantities of material, and the need for aflatoxins B_1, B_2 and G_1 in gram quantities has led us to develop a convenient, rapid and safe method of aflatoxin purification, by chromatography on the Waters Prep LC-500 instrument.

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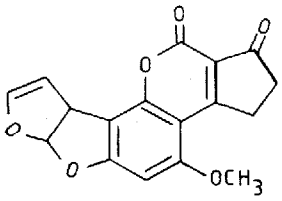

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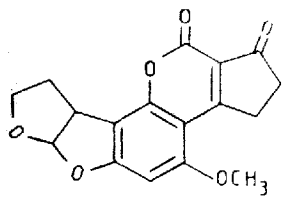

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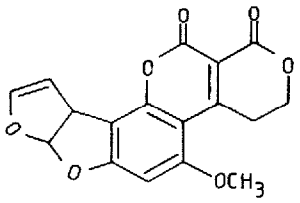

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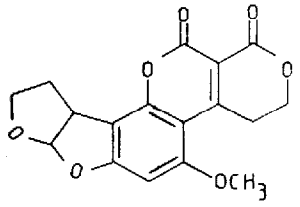

(4)

EXPERIMENTAL

Chemicals and equipment

All solvents used (ethyl acetate, chloroform, methanol, hexane and acetone) were of technical grade, purified by fractional distillation. For gravity column chromatography, Kieselgel 60 (particle size 0.063–0.200 mm) and Aluminiumoxid 90 (particle size 0.063–0.200 mm, Activity II–III) (E. Merck, Darmstadt, F.R.G.) were used. TLC was performed on Merck plates pre-coated with Kieselgel 60 plus a fluorescent indicator to a thickness of 0.25 mm (silica gel 60F-254) developed with chloroform–methanol (97:3, v/v) or chloroform–acetone (9:1, v/v). The conditions used for purification on the Prep LC-500 instrument (Waters, Milford, MA, U.S.A.) were as follows: column, two silica cartridges (250 g); eluent, chloroform; chamber pressure, 35 p.s.i.; solvent pressure, 25 p.s.i.; detector, refractive index; relative response, 20; flow-rate, 100 ml/min; chart speed, 5 min/cm.

Aflatoxin production

Aspergillus flavus (CSIR 840) was grown at 27°C for 5 days in stationary culture in the M_1 medium (47 l) consisting of sucrose (200 g), yeast extract (20 g), $MgSO_4 \cdot 7H_2O$ (10 g), $ZnSO_4 \cdot 7H_2O$ (26 mg), $CuSO_4 \cdot 5H_2O$ (2.6 mg), $Co(NO_3)_2 \cdot 6H_2O$ (1.3 mg) and $FeSO_4$ (5 mg) (per litre) in 500-ml flasks each containing 100 ml of medium. In preliminary growth experiments, this strain had been found to produce high levels of aflatoxins B_1 and G_1 and relatively smaller quantities of aflatoxin B_2, and practically no aflatoxin G_2. Ethyl acetate (100 ml) was added to each flask of the fungal broth in order to kill spores before filtration. The resultant fractions were treated separately.

Preliminary work-up

Media fraction. This fraction was separated into two phases. The aqueous phase was extracted with ethyl acetate (2 × 5 l) and the organic phases were pooled. The aqueous phase was adjusted with sodium hydroxide pellets to pH 10, to destroy unextracted aflatoxins, and then discarded. The organic phase was dried (sodium sulphate), filtered and evaporated. The residue was partitioned between chloroform and water (4 l, 1:1, v/v) and the aqueous phase was again treated with sodium hydroxide before it was discarded. The chloroform phase was filtered to remove crystalline material (Kojic acid), dried (sodium sulphate), filtered and evaporated to give a solid residue (21.0 g) hereafter referred to as fraction A.

Mycelial fraction. The mycelial material was homogenized for 5 min in a Waring blender with chloroform-methanol (1 l, 1:1, v/v). The resultant slurry was filtered, and the mycelial fragments were again homogenized using the same conditions. After filtration, the filtrates were pooled and evaporated and the mycelial remains were treated with 1 *M* sodium hydroxide (1 l) before they were discarded. The residue was partitioned between 90% methanol and *n*-hexane (1 l, 1:1, v/v), and the 90% methanol layer was evaporated. The residue was partitioned between chloroform and water (1 l, 1:1, v/v); the chloroform layer was dried (sodium sulphate), filtered and evaporated to give a solid residue (9.7 g) hereafter referred to as fraction B. The *n*-hexane and water fractions were treated with sodium hydroxide before they were discarded.

Preliminary clean-up

Fractions A and B were pooled and purified on a silica gel gravity-fed column (10 cm I.D.) (1.7 kg silica) and eluted with chloroform–acetone (10 l) (95:5, v/v). The eluates, hereafter referred to as fractions C (11.3 g, containing aflatoxins B_1 and B_2, as well as several unidentified pigments) and D (3.2 g, containing aflatoxins B_1, B_2 and G_1 but no pigments) were collected and evaporated to dryness. Fraction C was further purified by chromatography on alumina (600 g), eluting with chloroform–acetone (80:20, v/v) to give a Fraction E (1.18 g, containing only aflatoxins B_1 and B_2) as a cream coloured powder.

Purification on Waters Prep LC 500

Fraction E. The entire fraction in chloroform (5 ml) was purified by a single passage through the system at a flow-rate of 100 ml/min. When the refractive index detector indicated the beginning of the aflatoxins peak (about 8 min), 50-ml fractions were collected and monitored by TLC[4]. In this way, pure aflatoxin B_1 (700 mg), pure aflatoxin B_2 (80 mg) and a mixture of aflatoxins B_1 and B_2 (320 mg) were obtained. The mixture was hydrogenated to give pure aflatoxin B_2 (280 mg)[5].

Fraction D. The entire fraction in chloroform (5 ml) was purified by a single passage through the Waters system at a flow-rate of 100 ml/min. The chromatographic trace is shown in Fig. 1. Collection of 50-ml fractions, as above, monitored by TLC, gave pure aflatoxin B_1 (390 mg), pure aflatoxin G_1 (2.1 g), a mixture of aflatoxins B_1, $B_2$and G_1 (150 mg) and a mixture of aflatoxin B_1 and an impurity of lower R_F (450 mg). Total yields of aflatoxins: B_1 (1.09 g), B_2 (360 mg), G_1 (2.1 g). The purity of the aflatoxins was checked by elemental analysis, and by ^1H and ^{13}C NMR spectroscopy at 500 and 125 MHz, respectively.

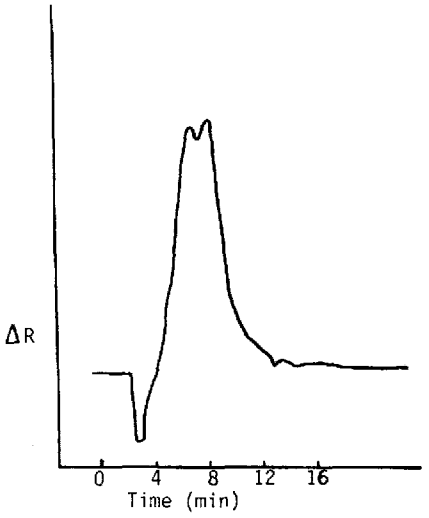

Fig. 1. Purification of fraction D on the Waters Prep 500. ΔR is the difference in refractive index between the column effluent and the eluting solvent.

Precautions

Standard precautions necessary when working with aflatoxins were observed. These included the mandatory use of gloves and protective clothing, the performance of all work involving fungal extractions in a fume-hood and the washing of all used glassware and benchtops with sodium hypochlorite solution.

DISCUSSION

The above methodology provides easy access to large quantities of aflatoxins B_1, B_2 and G_1. The method is rapid and convenient, and obviates the use of preparative TLC plates, which becomes cumbersome with larger amounts of material. Moreover, purification with the Prep LC-500 system also removes the need for scraping off bands from the preparative plates and the concomitant risk of breathing in silica impregnated with aflatoxins. With the recycle facility available on the machine, the method can easily be scaled up for even larger amounts of sample.

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