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Production of aflatoxin B_1 by *Aspergillus ruber* THOM and CHURCH

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Abstract

Production of aflatoxins by *Aspergillus ruber* THOM and CHURCH was first reported by KULIK and HOLADAY (1967), although these results have lacked confirmation. In this paper we provide evidence that this fungal strain produces aflatoxins. This finding has implications for food hygiene, especially in countries where such moulds are used in the preparation of foodstuffs.

Introduction

The principal fungal strains producing aflatoxins belong to the species *Aspergillus flavus* LINK and *Aspergillus parasiticus* SPEARE [1]. Various other species have been suspected to form these toxins. They include *Aspergillus niger* V. THIEGH, *Aspergillus wentii* WEHMER, *Penicillium citrinum*, *Penicillium variabile* [3, 4] and *Aspergillus ruber* THOM and CHURCH [3].

In the course of a study of the fungistatic action of phosphine for conservation of foodstuffs [5] chromatographic analysis (HPLC) of cultures of *Aspergillus ruber*, *Aspergillus chevalieri* and *Aspergillus repens* detected substances with the same characteristics as aflatoxins B_1, B_2, G_1 and G_2.

The present study was designed to find out whether aflatoxin B_1 was in fact produced by one of the suspected fungal strains of *Aspergillus ruber* B 38 C [5].

Material and methods

Aflatoxins

Standard aflatoxin B_1 (Sigma, St. Louis, USA) was dissolved in benzene/acetonitrile (90/10) at a concentration of 10 μg/ml.

Strains

The different strains of *Aspergillus ruber* isolated from various foodstuffs were kindly provided by the Department of Microbiology (INSERM, Le Vesinet, France). The purity and identity of each strain were checked using the morphological criteria described by RAPER and FENNEL [8].

Cultures

Strain B 38 C was cultured in 500 ml Erlenmeyer flasks on the following medium (50 ml): yeast

extract (2%), sucrose (20%), $MgSO_4$ (1%), $FeSO_4$ (0.05%). After inoculation of the medium in each flask with 1 ml of the spore suspension from a 3 week old culture, the flasks were incubated for 14 days at 25 °C as stationary cultures.

Control

This experiment was run three times with clean and carefully rinsed by chloroform glassware. For each one, a blank series (uninoculated medium) was done in the same way.

Extraction procedure

Using an A.O.A.C. method [6], aflatoxin B_1 was extracted with chloroform from 3 combined cultures. The extracts were filtered (Whatman No. 1), and purified on a silica gel column (G 60 Merck). Aflatoxin B_1 was eluted with chloroform/methanol (97/3), evaporated to near dryness, transferred to small vials in chloroform, and then evaporated to complete dryness under nitrogen. One half mililiter of benzene/acetonitrile (90/10) were added to the dry residue in each of the vials, which were then shaken in a vortex shaker.

Thin layer chromatography

Further purification was carried out on preparative thin layer chromatography plates (silica gel G 60, 20 × 20 cm Merck 5553). After development in the solvent mixture diethyl oxide/methanol/water (94/4.5/1.5), the blue fluorescent bands with the same Rf as the known standard (run on identical plates under the same conditions) were scraped into tubes containing chloroform/methanol (97/3). The tubes were shaken, and then centrifuged at 10000 rpm.

Two other successive purifications with the same process, using chloroform/acetone (90/10) and secondly toluene/ethyl acetate/formic acid (5/4/1) were necessary to obtain a clear separation of the suspect band. The final extract was evaporated under nitrogen, and taken up in 1 ml of chloroform.

Confirmatory tests

Various tests of increasing specificity were carried out. Three analytical TLC plates, spotted with the extract and aflatoxin B_1 standard were developed in the solvent system: diethyl oxide/methanol/water (94/4.5/1.5). One plate was sprayed with 25% sulfuric acid in methanol [9], one with 5%

Table 1. Chromatographic evidence for the presence of aflatoxin B_1 in extracts of *Aspergillus ruber* strain B 38 C.

TLC	TLC	TLC	TFA derivation
Ethyl ether 94	Chloroform 90	Toluene 5	Chloroform 46
Methanol 4.5	Acetone 10	Ethyl acetate 4	Methanol 4
Water 1.5		Formic acid 1	Acetic acid 1
			Water 0.4
Rf B38C 0.543	Rf B38C 0.425	Rf B38C 0.343	Same Rf as B_1 0.527
Rf B_1 0.543	Rf B_1 0.425	Rf B_1 0.3433	Same Rf as derivatized B_1 = 0.224

Spray with 25% méthanol sulfuric acid	Spray with 5% nitric acid	Spray with 10% sodium hypochlorite
Blue fluorescence turns yellow identical to aflatoxin B_1 standard.	Blue fluorescence turns yellow identical to aflatoxin B_1 standard.	Disappearance of fluorescence in extract and standard.

nitric acid [2], and the third with 10% sodium hypochlorite [10].

The test described by PRZYBYLSKI [7] was carried out as follows: 2 spots of fungal extract and 2 spots of aflatoxin B_1 standard were placed on a 10 × 10 cm TLC plate. One of each (extract and standard) was treated with trifluoroacetic acid (TFA). After migration in the solvent mixture chloroform/methanol/acetic acid/water (46/4/1/0.4), the Rf of the derivatized and underivatized metabolites were compared to standards (aflatoxin B_1 and aflatoxin B_{2a}).

Finally, the purified extract and known standard were analyzed by UV spectroscopy (Bausch & Lomb 2000) and mass spectrometry (electron impact ionization and electron desorption; Finigan, West Germany).

Results and discussion

The results of the various chemical and chromatographic analyses are shown in Table 1. The extract behaved identically to the standard aflatoxin B_1. In the blank series, no suspect spot was observed.

The UV spectrum of the purified extract of B 38 C is identical to that of the aflatoxin B_1 standard (Fig. 1).

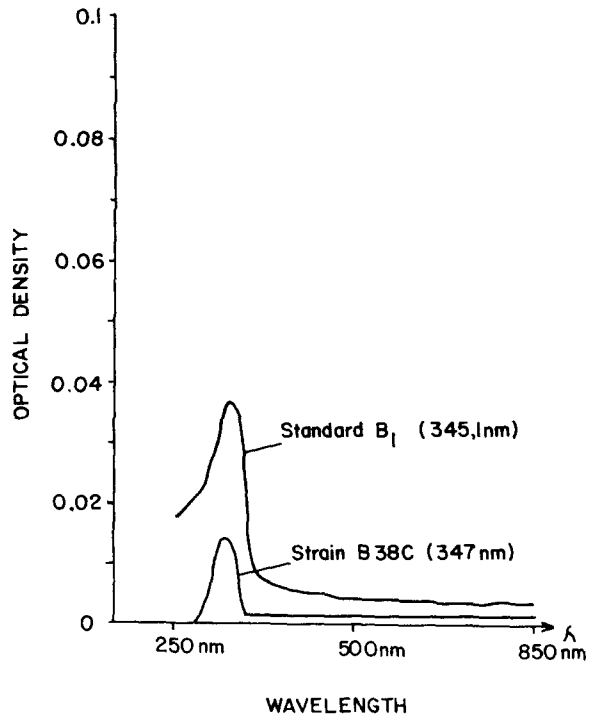

Fig. 1. UV absorption spectra of aflatoxin B_1 standard and the extract of *Aspergillus ruber* strain B 38 C.

Electron impact mass spectrometry showed that the fungal extract had the same ion fragments as the aflatoxin B_1 standard as well as the same parent peak (312 Daltons). These results were confirmed by the electron desorption technique (Fig. 2).

The results of this study confirm the findings of KULIK and HOLADAY [3] and our previous HPLC results [5] showing the presence of peaks corresponding to aflatoxins B_1, B_2, G_1 and G_2 in extracts of *Aspergillus ruber*. Although the results here only confirmed the presence of aflatoxin B_1, the most toxic member of this group of mycotoxins, it is likely that the other peaks corresponded to the other aflatoxins.

Although these experiments were only carried out on one strain (*Aspergillus ruber* B 38 C), the other strains probably also produce aflatoxins, because similar peaks were detected by HPLC analysis of their culture extracts.

Despite the low potential for production of toxins by these strains, the risk of aflatoxin formation in moist foodstuffs (cereals, husks, etc.) should not be underestimated, especially in view of the widespread presence of xerophilic strains of *Aspergillus ruber* of the in such foodstuffs.

Moreover, the use of these micromycetes in the preparation of food in some parts of the world (Far East, *) indicates a need for strict monitoring of such foodstuffs due to the carcinogenicity of aflatoxin B_1.

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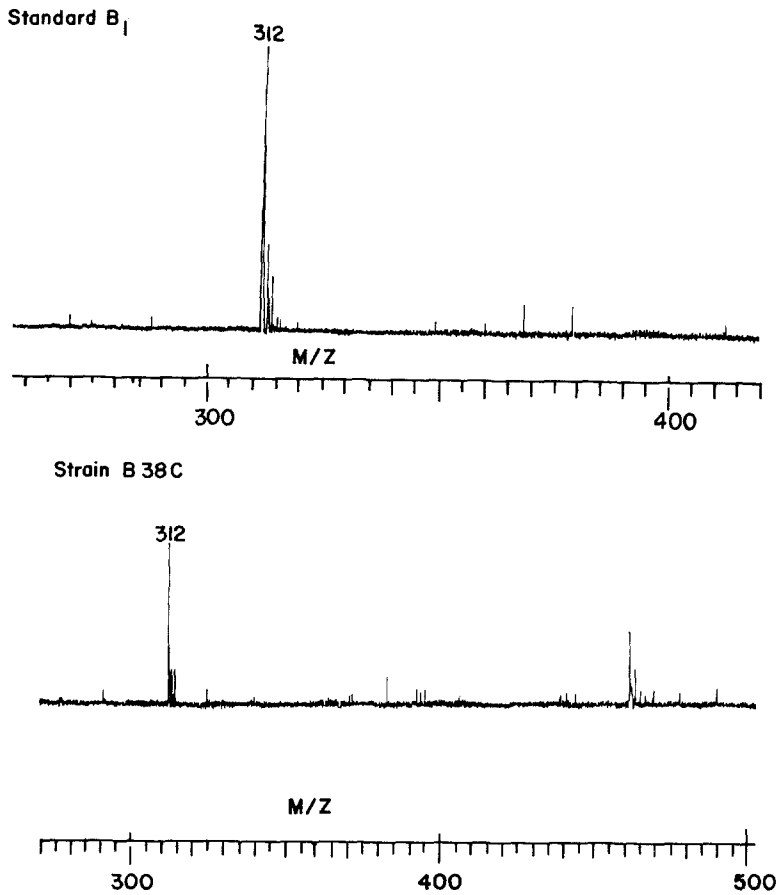

Fig. 2. Mass spectra (electron desorption) of aflatoxin B_1 standard and the extract of fungal strain B 38 C.

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