Developments in Food Science

8

MYCOTOXINS
Production, Isolation, Separation and Purification

Edited by
VLADIMÍR BETINA

ELSEVIER

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Vladimír Betina

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PREFACE

Mycotoxins are important fungal metabolites that have been recognized within the last two decades as a potential threat to human and animal health, and the effects of mycotoxins result in economic losses to various extents (e.g. aflatoxins, trichothecenes). Mycotoxins present a challenge for scientists working in a wide range of disciplines such as microbiology, biochemistry, structural chemistry, toxicology, pharmacology and genetics.

The evaluation of the role and extent of mycotoxin contamination in feedstuffs and foods requires the isolation of the toxin(s) from a suspected commodity or from fungal cultures isolated from the suspected material. This book has been written with the aim of helping investigators in the field by bringing together the most important data, scattered in the literature, on the production, isolation, purification and characterization of the best known mycotoxins. Expert scientists in the field of mycotoxin isolation and characterization have provided contributions based on extensive personal experience.

The book consists of two parts. In the General Part, data on the occurrence, production, biological activities and biochemical modes of action of mycotoxins are discussed, followed by chapters describing screening for toxigenic fungi and applications of chromatographic techniques in the isolation, purification and characterization of mycotoxins.

In the Special Part, key chemical families and the most important individual mycotoxins have been chosen, the largest families being the trichothecenes, the epipolythiodioxopiperazines and the cytochalasans. As would be expected, ergot alkaloids and toxins from Basidiomycetes have not been included. Data on producing organisms and the physical, chemical and biological properties of each mycotoxin are given, followed by methods of production, isolation, purification, chromatographic characterization, detection and assays.

I express my thanks to all of the contributors for their excellent cooperation in preparing their manuscripts.

Bratislava
December 1983

Vladimír Betina

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CHAPTER 1

TOXIGENIC FUNGI AND THEIR OCCURRENCE

V. BETINA

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1.1. INTRODUCTION

The development of man has been associated with various uses of fungi. The age-old role of these microorganisms in the production of bread, alcoholic beverages or Oriental food fermentations is well known. Today, fungi are applied in the production of cheese, livestock feeds, enzymes, organic acids, vitamins, antibiotics and steroids, and also have a substantial role in plant, animal and human diseases and in crop and food spoilage. However, mycotoxins and mycotoxicoses received little attention until the early 1960s, when the aflatoxins were discovered. Mycotoxicoses are defined as "poisonings of the host which follow the entry into the body of toxic substances of fungal origin"[1].

The oldest known mycotoxicoses are associated with various species of the parasitic genus *Claviceps* and their metabolic products. The toxic effects of these compounds have been the cause of mass poisonings in both man and animals over several centuries. The animal poisonings usually resulted from feeding on grass infected with the ergot fungus, whereas the toxic effects in man stemmed from eating bread of other products made from rye flour which was contaminated with sclerotia of *Claviceps purpurea*[2].

Outbreaks of other mycotoxicoses have been reported in many countries, such as diseases of pigs and horses associated with the ingestion of barley infected with *Fusarium graminearum* (barley scab)[3] in the U.S.A.; stachybotryotixicosis of horses in the U.S.S.R., first reported in the 1930s, which occurred as a result of the fungus *Stachybotrys atra* growing on straw[1]; facial eczema in sheep in New Zealand that had grazed on pasture infected with the fungus *Pithomyces chartarum*[4], from which sporidesmin was isolated[5]; liver tumours induced by yellowed rice toxins in Japan[6]; and alimentary toxic aleukia (ATA) reported in eastern Siberia in 1913 and caused by fusaria growing on grain that had over-wintered on the ground[7].

1.2. TOXIGENIC FUNGI

Toxigenic species can be found in all major taxonomic groups of fungi. However, most of the mycotoxins hitherto known have been recognized as metabolic products of genera such as *Aspergillus*, *Penicillium* and *Fusarium*. In addition to the already mentioned *Claviceps*, other *Ascomycetes* are known to produce various mycotoxins. Examples of toxigenic fungi belonging to several taxonomic groups and their mycotoxins are given in Tables 1.1, 1.2 and 1.3. The mycotoxins listed are far to be complete. Toxins from *Basidiomycetes* are not included in this book.

As mycotoxin-producing fungi are abundant in nature and favourable conditions for their production are easily achieved, it was concluded[8] that few edible substances are considered safe from mycotoxin contamination. Fungal invasion of foods can occur at any stage of the food production chain, i.e., from the time of planting through harvesting, transportation and storage, during the prepara-

TABLE 1.1

MYCOTOXINS PRODUCED BY SPECIES OF THE GENUS *ASPERGILLUS*

Mycotoxin	Producing organism	Mycotoxin	Producing organism
Aflatoxins	*A. flavus*, etc.	Fumitremorgins	*A. fumigatus*
Aflatrem	*A. flavus*	Helvolic acid	*A. fumigatus*
Ascladiol	*A. clavatus*	Kojic acid	*A. fumigatus*, etc.
Aspergillic acid	*A. flavus*	Malformins	*A. niger*
Citreoviridins	*A. terreus*	Ochratoxins	*A. ochraceus*
Cyclopiazonic acid	*A. flavus*	Patulin	*A. clavatus*, etc.
5,6-Dimethoxy-sterigmatocystin	*A. multicolor*	Physcion	*A. glaucus*
Emodin	*A. wentii*	Sterigmatocystin	*A. versicolor*
Erythroglaucin	*A. glaucus*	Terretonin	*A. terreus*
Flavutoxin	*A. flavus*	Viomellein	*A. ochraceus*
Fumagillin	*A. fumigatus*	Xanthocillin X	*A. chevalieri*
Fumitoxins	*A. fumigatus*	Xanthomegnin	*A. ochraceus*

TABLE 1.2

MYCOTOXINS PRODUCED BY SPECIES OF THE GENUS *PENICILLIUM*

Mycotoxin	Producing organism	Mycotoxin	Producing organism
Brevianamide A	*P. viridicatum*	Penicillic acid	*P. puberulum*, etc.
Cyclochlorotine	*P. islandicum*	Penitrems	*P. crustosum*
Cyclopenin	*P. viridicatum*	PR toxin	*P. roqueforti*
Citreoviridin	*P. citreo-viride*	Roquefortine	*P. roqueforti*
Citrinin	*P. citrinum*, etc.	Rubratoxins	*P. rubrum*
Erythroskyrine	*P. islandicum*	Rubrosulphin	*P. viridicatum*
Griseofulvin	*P. griseofulvum*	Rugulosin	*P. rugulosum*
Islanditoxin	*P. islandicum*	Secalonic acids	*P. oxalicum*
Janthitrems	*P. janthinellum*	Verruculogen	*P. verruculogenum*
Luteoskyrin	*P. islandicum*	Viomellein	*P. viridicatum*
Mycophenolic acid	*P. brevi-compactum*, etc.	Viridicatin	*P. viridicatum*
		Viridicatumtoxin	*P. viridicatum*
β-Nitropropanoic acid	*P. atrovenetum*	Xanthocillin X	*P. notatum*
		Xanthomegnin	*P. viridicatum*
Patulin	*P. urticae*, etc.	Xanthoviridicatins	*P. viridicatum*
Paxilline	*P. paxilli*		

TABLE 1.3

MYCOTOXINS PRODUCED BY VARIOUS GENERA

Mycotoxin	Producing organism	Mycotoxin	Producing organism
Alternariol	*Alternaria* spp.	Paspalicine	*Claviceps paspali*
Butenolide	*Fusarium tricinctum*	Paspaline	*Claviceps paspali*
Chaetoglobosins	*Chaetomium globosum*	Patulin	*Byssochlamys fulva*
Cytochalasins	*Phoma exigua*, etc.	Tenuazonic acid	*Alternaria* spp.
Diplodiol	*Diplodia macrospora*	Trichothecenes	*Fusarium*, *Myrothecium*, etc.
Mollicellins	*Chaetomium mollicellum*	Zearalenone	*Gibberella zeae*

tion of foods in restaurants and homes, and until the food is consumed. It should be added that mycotoxin-contaminated feed has caused serious economic losses, of which the Turkey X disease is a well known example.

1.3. NATURAL OCCURRENCE OF TOXIGENIC FUNGI AND MYCOTOXINS

Because mycotoxins belong to environmental chemicals which can poison both man and animals (and in some instances plants), their occurrence and also the occurrence of their producing fungal strains have been studied by many workers. Toxigenic fungi can grow and produce their toxins on growing crops, on leaves and stems, on grains and seeds, on meat and milk products, etc. In order to demonstrate the importance of the natural occurrence of toxigenic fungi and their toxins, this section presents a selection of data from the literature. More systematic studies of this topic have been published elsewhere[9-11].

The production of aflatoxins by *Aspergillus flavus* strains on agricultural crops such as groundnut, cottonseed, wheat, rice, barley, coconut, corn, dried peas, oat, sweet potato, millet and cassava is well documented[12]. Several mycotoxins, including citrinin, zearalenone and trichothecenes, have been found in cereals. Some examples are given below. Citrinin was isolated from a strain of *Penicillium viridicatum* from a batch of barley which, on ingestion by pigs and rats, caused chronic kidney degeneration[13].

Many trichothecene mycotoxins, when produced by *Fusaria* either in vitro or naturally, cause emesis in a variety of laboratory animals. Morooka et al.[14] isolated from fusaria-infected barley a trichothecene toxin. Yoshizawa and Morooka[15] described its structure and assigned a new trivial name, 4-deoxynivalenol. Vesonder et al.[16] isolated vomitoxin from *Fusarium*-contaminated field corn. Deoxynivalenol and vomitoxin are identical[17].

Vomitoxin at levels ranging from 0.5 to 10 $\mu g\ g^{-1}$ was found in 24 of 52 pre-harvest corn samples collected in mid-October, 1977, from farms in north-west Ohio[18]. Ellison and Kotsonis[19] presented evidence that T-2 toxin was another emetic factor in mouldy corn. Of those fungi which produce T-2 toxin, *Fusarium tricinctum* has been recognized as particularly important with regard to mouldy corn toxicosis in the mid-western U.S.A.[20]. A sample of poefusarin (a mycotoxin suspected of being one of the toxins involved in alimentary toxic aleukia) contained T-2 toxin in an amount sufficient to explain the toxicity of the sample. In addition, neosolaniol, T-2 tetraol and zearalenone were present in the sample[21].

The highest known oesophageal cancer rate in Africa occurs in the south-western districts of the Republic of Transkei. Corn is the main dietary staple in the area. Two *Fusarium* toxins, deoxynivalenol and zearalenone, were detected at biologically significant levels in hand-selected, visibly *Fusarium*-infected corn kernels. The level of natural contamination of corn kernels with both mycotoxins was considerably higher in the high-incidence area of oesophageal cancer than in the north-eastern region of the country, where the cancer rate is relatively low[22].

Citrinin, implicated in porcine nephropathy[13], has been found as a natural contaminant of corn, rice, wheat, rye, barley and oats[23].

A wide variety of foodstuffs, including rice, soybeans, wheat, bread, flour, white and black peppercorn, beans and groundnuts support the growth of the storage mould *Penicillium islandicum*. Out of 385 samples of soybeans collected from the U.S. market, 94 were contaminated with this fungus. A large number of toxic and non-toxic metabolites are known to be produced by *P. islandicum*. Among the potent toxins are luteoskyrin, cyclochlorotine, islanditoxin and simatoxin[24].

Aspergillus flavus readily grows on groundnuts and grains when they are stored traditionally in the open, in hot, humid conditions. Approximate relationships have been reported between aflatoxin contamination of market food samples and the incidence of primary liver cancer in Uganda, Swaziland and Thailand. A significant correlation between the incidence of liver cancer and the amount of aflatoxin actually consumed in three different areas in Kenya has been reported[25].

Toxic fungi have been shown to occur in a variety of nuts (pistachio nuts, chestnuts). Isolates of known toxigenic fungi from pistachio nuts in Iran included *Aspergillus flavus, A. ochraceus, A. versicolor* and others. All isolates of *A. flavus* and *A. parasiticus* produced aflatoxin B_1 and the isolates of *A. versicolor* and *A. nidulans* produced sterigmatocystin[26].

Emodin, a toxic fungal metabolite which is regarded as a precursor of many fungal anthraquinones, was characterized by Wells et al.[27] as a metabolite of *Aspergillus wentii* isolated from weevil-damaged chestnuts.

Because of their high protein content and relatively low cost, dried beans are consumed in substantial amounts. Mislivec et al.[28] evaluated the potential for mycotoxin production by moulds in dried beans. The flora was dominated by species of the *Aspergillus glaucus* group, the toxigenic species *A. ochraceus, Penicillium cyclopium* and *P. viridicatum*, and species of *Alternaria, Cladosporium* and *Fusarium*. Of 209 species of *Aspergillus* and *Penicillium* screened for mycotoxin production on sterile rice substrate, 114 produced one or more of the following mycotoxins: *A. flavus*, aflatoxins; *A. ochraceus*, ochratoxins; *A. nidulans, A. unguis* and *A. versicolor*, sterigmatocystin; *P. cyclopium*, penicillic acid; *P. citrinum* and *P. viridicatum*, citrinin; and *P. urticae*, patulin and griseofulvin.

The widespread occurrence of ochratoxin-producing fungi, their ability to grow on a variety of economically important feeds and foodstuffs and the natural occurrence of ochratoxin constitute a threat to both animal and public health[29].

Chemically related tremorgenic mycotoxins have been discovered in recent years in fungi isolated from ryegrass pastures in New Zealand, Australia and North America. These toxins include verruculogen, fumitremorgins, paxilline and janthitrems[30-32].

An isochroman mycotoxin was isolated from *Penicillium steckii* from mouldy millet hay suspected of causing deaths in cattle[33]. The practice of baling hay in large round or square bales has become popular. However, as these bales are generally stored in the open, they become contaminated with fungi. A strain of *Aspergillus terreus* was isolated from a bale (*A. terreus* is known to produce the toxins citrinin and patulin) and from this strain a new toxic metabolite, terretonin, was isolated[34]. From extracts of cultures of *Aspergillus fumigatus* isolated from silage, new mycotoxins called fumitoxins were isolated in France[35].

The formation of cytochalasin B by a strain of *Hormiscium* sp. in tomatoes[36] and in potatoes by strains of *Phoma exigua* var. *exigua*[37] has been reported. Among mould isolates from decaying tomato fruit, toxigenic strains were isolated and their toxicity was attributed to tenuazonic acid (*Alternaria alternata*), T-2 toxin (*Fusarium sulphureum*), citrinin and patulin (both from *Penicillium expansum*)[38].

Bacon et al.[39] demonstrated the ability of *Aspergillus ochraceus* to colonize poultry feed and produce penicillic acid and ochratoxin A at favourable temperatures and moisture levels.

Trichothecenes are produced by various species of fungi from the genera *Fusarium, Trichothecium, Myrothecium, Trichoderma, Cephalosporium, Verticimonosporium* and *Stachybotrys*. As mentioned above, these fungi attack many agricultural plant products. In our laboratory, from an apple with a bitter taste a strain of *Trichothecium roseum* was isolated which produced trichothecin and related secondary metabolites[40].

Byssochlamys fulva and its imperfect state *Paecilomyces varioti* have been isolated from processed fruits, cassava bread, sorghum brandy and Manitoba butter[41]. Various metabolites of *B. fulva* (*P. varioti*) have been reported, including the mycotoxins byssochlamic acid, byssotoxin A and patulin. Patulin is also produced by several fungi of *Aspergillus* and *Penicillium*. *P. expansum*, owing to its ability to grow at $0^{o}C$, is a major cause of loss of apples and pears in storage. Twenty-seven isolates of *P. expansum* obtained from Europe, Australia and North America from seven different fruit hosts all produced patulin in culture[42]. Patulin has been found in apple fruits[43] and in apple juice[44]. Wilson and Nuovo[45] found patulin in all of 60 cultures tested (all isolated from apples). Scott and Somers[46] observed stability of patulin and penicillic acid in fruit juices and flour.

While sun-drying on the tree, fig fruits are particularly vulnerable to infection and colonization by *Aspergillus flavus*. Aflatoxin accumulation equaled levels frequently reported for groundnuts and cereal grains[47].

Aspergillus oryzae is widely used for the preparation of koji, a starter in the production of soy sauce, and miso, a fermented soybean paste used in Japanese cooking (900,000 metric tons annually). Miso is prepared by first inoculating rice with *A. oryzae*, a combination called koji-rice, which produces an enzyme source for subsequent admixture with soybeans. The process is analogous to that in which malt is used in brewing. The subsequent soybean-koji-rice fermentation is conducted at 27°C for 50 h. As this fungus was widely used in food processing, the organism was thoroughly investigated for production of toxins. El-Hag and Morse[48] reviewed papers by other workers who had all concluded that *A. oryzae* was not an aflatoxin producer. The former authors, however, found that a variant of *A. oryzae* approved for use in food processing is variable and has a propensity to produce aflatoxin on other substrates.

The simultaneous production of penicillic acid and patulin by an atypical strain of *Penicillium roqueforti* isolated from cheddar cheese was reported by Olivigni and Bullerman[49]. *P. roqueforti*, the essential fungus used in the manufacture of roquefort and other varieties of blue cheeses, has been shown to produce other toxic compounds. These include PR toxin[50] and roquefortine, which has been detected in blue cheese samples from different countries[51]. Orth[52] reported finding PR-toxin-producing *P. roqueforti* isolated in nut ice cream, a dessert cream and on a red wine bottle cork. The significance of these toxins in terms of human health is unclear, as blue-veined cheeses have been consumed for centuries without apparent ill-effects. However, the potential carcinogenic nature of penicillic acid and patulin[53] possibly makes them a cause for concern.

Cyclopiazonic acid production by *P. camemberti* and the natural occurrence of this mycotoxin in cheese has also been reported[54], together with other findings of penitrem A[55] and sterigmatocystin[56].

Several investigators have isolated toxigenic fungi from meat products. Halls and Ayres[57] reported that isolates of *Aspergillus versicolor* from country-cured ham were capable of producing sterigmatocystin. Three of these isolates were tested and found to produce sterigmatocystin on country-cured ham. Toxigenic aspergilli and penicillia were isolated and identified from country-cured hams and fermented sausages by other workers. Aflatoxin-, ochratoxin-, strigmatocystin- and citrinin-producing strains were isolated from country-cured hams. Wu et al.[58] isolated cultures of *Aspergillus* and *Penicillium* from aged, cured meats and tested their toxicity to chicken embryos. Several isolates of *A. ruber*, *A. repens*, *A. sydowi*, *A. restrictus*, *A. amstelodami*, *A. chevalieri* and *A. fumigatus* exhibited toxicity. Similarly, some isolates of *P. notatum*, *P. brevicompactum* and *Penicillium* spp. were found to be highly toxic. In these investigations, there was no direct or indirect evidence that aged, cured meats contain toxic metabolites.

However, two strains of *A. ochraceus* isolated from country-cured hams produced ochratoxin A and B on rice, defatted groundnut meal and country-cured ham. After incubation of ham for 21 days, one third of the toxin was found in the mycelial mat on the ham surface, whereas two thirds had penetrated into the meat[59].

From contaminated candy, we isolated a toxigenic fungus identified as *Aspergillus repens*[60]. Apart from antibacterial and antifungal activity, crude mycelial extracts of this fungus were toxic to *Artemia salina* larvae and HeLa cells. Of the six metabolites, isolated and identified as echinulin, physcion, erythroglaucin, flavoglaucin, asperentin (cladosporin) and asperentin 8-methyl ether, the broadest activity spectrum was displayed by asperentin. The latter, at a concentration of 86 $\mu g\ ml^{-1}$, caused 50% mortality in *A. salina* larvae. The highest toxicity towards HeLa cells was found in physcion, which caused 50% growth inhibition at a concentration of 0.1 $\mu g\ ml^{-1}$.

We could conclude that the natural occurrence of toxigenic fungi is very broad. However, one has to distinguish between the natural occurrence of their toxins and the production of these toxins under laboratory conditions. The best conclusion seems to be the following statement concerning the natural occurrence of aflatoxins (appliable also to other mycotoxins) in a recent book[61].

"Aflatoxins arise naturally when a toxin-producing strain of *Aspergillus flavus* or *A. parasiticus* grows on a substrate in a geographical area where environmental conditions are suitable for the development of the mould. Many strains of *A. flavus* do not produce aflatoxins, so the mere presence of mouldiness is not, of itself, indicative of toxin production. The natural occurrence of aflatoxins can only be described as such when the initial inoculation by the mould spores and subsequent mould development are a natural sequence of events. In view of the fact that the *A. flavus* group of moulds is ubiquitous and capable of development over a wide range of temperature on substrates of high carbohydrate content, agricultural commodities and their products are most vulnerable to contamination. Because of this vulnerability a world-wide effort, involving scientists of many disciplines, was initiated to establish the conditions under which *A. flavus* produces aflatoxins. Research was also directed to establishing what foods are potentially at risk to *A. flavus* infection during their production. Much research work has entailed the deliberate infection of commodities with isolates of *A. flavus*, and it is important to make a distinction between this method of aflatoxin production and the natural occurrence of the toxin referred to above".

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