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Degradation of ochratoxin A by *Aspergillus* species

János Varga[a,*], Krisztina Rigó[a], József Téren[b]

[a]*Department of Microbiology, Attila József University, P.O. Box 533, H-6701 Szeged, Hungary*
[b]*Animal Health and Food Control Station, P.O. Box 446, H-6701 Szeged, Hungary*

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Abstract

Mycotoxin contamination of agricultural products is a serious health hazard throughout the world. Besides attempts to eliminate mycotoxins from contaminated substrates by physical and chemical methods, the ability of microbes to degrade mycotoxins is now being widely examined. In this study, several *Aspergillus* species were examined for their ability to degrade ochratoxin A. *A. fumigatus* and black *Aspergillus* strains were found to detoxify ochratoxin A in culture media. The kinetics of ochratoxin A detoxification by an atoxigenic *A. niger* strain was examined by thin layer chromatography, high-performance liquid chromatography and an immunochemical technique. *A. niger* CBS 120.49 was found to effectively eliminate ochratoxin A from both liquid and solid media, and the degradation product, ochratoxin α, was also decomposed.

Keywords: Ochratoxin A; Detoxification; Ochratoxin α; *Aspergillus niger*; High-performance liquid chromatography (HPLC)

1. Introduction

Ochratoxins are mycotoxins which exhibit nephrotoxic, immunosuppressive, teratogenic and carcinogenic properties (Smith and Moss, 1985). Ochratoxin A (OA), the most potent, chlorinated derivative was discovered in 1965 as a secondary metabolite of an *Aspergillus ochraceus* strain (van der Merwe et al., 1965). In subsequent years several other *Aspergillus* and *Penicillium* species were described as producers of these toxins (for references, see Varga et al., 1996; Abarca et al., 1997). OA contamination of green coffee beans and other plant products such as barley, wheat, bread and spices is a serious health hazard throughout the world (Smith and Moss, 1985). Although prevention of growth and mycotoxin production of fungi on plants and in feedstuffs is usually considered as the best approach to impede the harmful effects of mycotoxins on animal and human health, detoxification of contaminated agricultural products is also of prime importance. OA is a moderately stable molecule which can survive most food processing, such as roasting, brewing and baking, to some extent (Krogh et al., 1974; Scott, 1996). Several chemical and physical methods such as hypochlorite treatment (Castegnaro et al., 1991),

*Corresponding author. Tel.: +36-62-544-515; fax: +36-62-544-823.
E-mail address: jvarga@bio.u-szeged.hu (J. Varga)

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ammoniation (Chelkowski et al., 1982) and heat treatment (Boudra et al., 1995) have been developed to detoxify OA in animal feeds. However, these methods have met with varying degrees of success, and none of them are recommended for practical detoxification of OA-contaminated grains and feeds (Scott, 1996). Other promising methods suggested recently include ozone (McKenzie et al., 1997) and alkaline hydrogen peroxide treatment (Fouler et al., 1994), and gamma irradiation (Refai et al., 1996). Alternatively, microbes or their enzymes can also be applied for mycotoxin detoxification; such biological approaches are now being widely studied (Sweeney and Dobson, 1998).

Here we describe the results of our survey of the OA detoxifying activities of several *Aspergillus* species. The kinetics of the degradative process have also been examined in an OA-decomposing *Aspergillus niger* strain.

2. Materials and methods

2.1. Strains

The *Aspergillus* strains examined are listed in Table 1. Strains were maintained on malt extract agar slants.

2.2. Screening for OA degradation

The strains were grown in 2 ml of YES (2% yeast extract, 15% sucrose) medium containing 2 μg ml^{-1} OA (Sigma). Test tubes were inoculated with a dense conidial suspension of the strains and incubated at 30°C for 10 days in the dark. OA was extracted with 2 ml of dichloromethane. One milliliter of the organic phase was evaporated to dryness and dissolved in 200 μl acetonitrile. Five microliters of the extracts were spotted on thin layer chromatography (TLC) plates and chromatographed as described previously (Téren et al., 1996).

2.3. Kinetics of OA degradation

For kinetic studies, 2 ml of liquid YES medium, or YES agar plates (20 ml per Petri dish) containing 2.5 μg ml^{-1} OA, were inoculated with 20 μl of a conidial suspension (10^7 conidia ml^{-1}) of *Aspergillus niger* strain CBS 120.49. The liquid cultures were grown for 1, 3, 5, 7 and 9 days in triplicate. The YES plates were incubated for 1, 2, 4, 5, 6 and 9 days and two agar-plugs were taken as a sample (about 1 ml in volume).

Ochratoxin A was extracted with 2 ml of dichloromethane, the organic phase was transferred to a clean tube, vortexed with 2 ml of 1% $NaHCO_3$ and centrifuged. The aqueous phase was acidified to pH 2 and OA was reextracted with an equal volume of dichloromethane. Aliquots (5–10 μl) of these extracts were applied to high-performance TLC (HPTLC) plates, developed and OA was identified as described previously (Varga et al., 1996).

2.4. HPLC analysis

The dichloromethane extracts used for HPTLC analyses were evaporated and redissolved in appropriate amounts of water–acetonitrile–acetic acid (99:99:2). The high-performance liquid chromatography (HPLC) equipment consisted of an S1100 solvent delivery system, an S5110 sample injector valve with a 20 μl loop (SYKAM GmbH, Germany) and a Linear Instruments Model 200 detector at 333 nm. BST Rutin C_{18} BD HPLC columns (250 × 4 mm, particle size 10 μm; BioSeparation Techniques, Budapest, Hungary) were used. OA was eluted with water–acetonitrile–acetic acid (99:99:2) as mobile phase at a flow-rate of 1 ml min^{-1}. OA (Sigma) and ochratoxin α prepared by acid hydrolysis of OA as described by Xiao et al. (1995) were used as standards.

3. Results

3.1. Screening of Aspergillus isolates for OA detoxifying activity

A total of 70 *Aspergillus* isolates representing six sections of the *Aspergillus* genus (Gams et al., 1985) were tested for their ability to degrade OA (Table 1). Among the species tested, only isolates of *Aspergillus fumigatus* and black Aspergilli could eliminate OA from the medium. An atoxigenic *A. niger* strain (CBS 120.49) was selected for further studies.

Table 1
Aspergillus strains examined for OA degradation activities

Species	Strain number and origin[a]	OA degradation[b]
Section Flavi		
A. albertensis	ATCC 58745	−
A. alliaceus	FRR 4340	−
A. flavus	NRRL 1957	−
A. muricatus	IMI 368521	−
A. nomius	IMI 331920	−
Section Fumigati		
A. auratus	NRRL 4379	−
A. aureolus	NRRL 2391	−
A. botucatensis	CMB FA 0672	−
A. brevipes	NRRL 2439	−
A. duricaulis	IMI 217288	−
A. fennelliae	NHL 2953	−
A. fennelliae	NRRL 5534	−
A. fennelliae	NRRL 5535	−
A. fischerianus	NRRL A-7223	−
A. fumigatus	SZMC FK3	−
A. fumigatus	NCAIM F 056	−
A. fumigatus	NRRL 163	−
A. fumigatus	SZMC 1012	−
A. fumigatus	SZMC 1058	−
A. fumigatus	SZMC 1180	+
A. fumigatus var. *ellipticus*	NRRL 5109	−
A. fumigatus mut. *helvola*	NRRL 174	+
A. fumigatus var. *acolumnaris*	NRRL 5587	+
A. hiratsukae	IMI 349860	−
A. hiratsukae	NRRL 3008	−
A. hiratsukae	NRRL 3009	−
A. multiplicatus	CBM FA 0710	−
A. paulistensis	CBM FA 0690	−
A. primulinus	CBM FA 0685	−
A. thermomutatus	NRRL 3946	−
A. quadricinctus	IMI 058374	−
A. spathulatus	NHL 2948	−
A. spinosus	NRRL 3435	−
A. paleaceus	NRRL 4652	−
A. tatenoi	CBM FA 0702	−
A. udagawae	CBM FA 0703	−
A. unilateralis	NRRL 577	−
Aspergillus sp.	FRR 1266	−
Aspergillus sp.	NRRL 4179	−
Aspergillus sp.	SZMC JV 3	−
Section Circumdati		
A. bridgeri	RMF 7745	−
A. campestris	IMI 259099	−
A. ochraceus	FRR 3815	−
A. ochraceus	FRR 3846	−
A. ochraceus	FRR 543	−
A. ochraceus	SZMC Z1	−
A. ochraceus	SZMC Z3	−
A. sclerotiorum	NRRL 4491	−
A. sepultus	ATTC 58705	−
A. sulphureus	IMI 211397	−

Table 1. Continued

Species	Strain number and origin[a]	OA degradation[b]
Section Nigri		
A. carbonarius	IMI 041875	−
A. japonicus	JHC 564	(+)
A. niger	From coffee beans, Brazil	−
A. niger	From chili pepper	−
A. niger	From curry powder	−
A. niger	From hazelnut	−
A. niger	JHC 607	(+)
A. niger	CBS 120.49	+
A. niger	From dried parsnip	−
A. niger	From sage	−
A. niger	From sage	−
Section Nidulantes		
A. nidulans	FGSC 513	−
A. nidulans	IMI 086806	−
A. rugulovalvus	IMI 136775	−
A. tetrazonus	NRRL 201	−
A. tetrazonus	NRRL 4992	−
A. violaceo-brunneus	IMI 061449	−
Section Versicolores		
A. obscurus	NCAIM F 6601189	−
A. versicolor	SZMC 560	−
A. versicolor	SZMC 581	−

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, MD, USA; CBM, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; FRR, CSIRO Food Research Culture Collection, North Ryde, New South Wales, Australia; FGSC, Fungal Genetics Stock Center, KS, USA; IMI, International Mycological Institute, Egham, Surrey, UK; JHC, J.H. Croft's fungal collection, University of Birmingham, UK; NCAIM, National Collection of Applied and Industrial Microorganisms, Horticultural University, Budapest, Hungary; NHL, National Institute of Hygienic Sciences, Tokyo, Japan; NRRL, Agricultural Research Service Culture Collection, Peoria, IL, USA; RMF, Rocky Mountain Herbarium, Fungi, University of Wyoming, Laramie, WY, USA; SZMC, Szeged Microbiological Collection, Szeged, Hungary.

[b] −, did not degrade OA; +, OA was completely eliminated from the medium; (+), OA was partially degraded.

3.2. Kinetics of OA decomposition by A. niger CBS 120.49

The kinetics of OA degradation of *A. niger* strain CBS 120.49 was examined in liquid and solid YES media. TLC analysis indicated that OA was degraded relatively slowly in liquid YES media; OA was completely converted to ochratoxin α within 7 days of incubation (data not shown). The amount of ochratoxin α increased for 6 days, then gradually decreased to trace amounts after 10 days' incubation (data not shown). OA was detoxified faster in solid media than in liquid media, as confirmed by both TLC and HPLC analyses (Figs. 1 and 2). The amount of OA decreased to less than 20% of the original amount (about 500 ng ml^{-1}) within 2 days, and OA was completely converted to the much less toxic ochratoxin α within 5 days in solid media (Figs. 1 and 2). A more sensitive immunochemical technique (detection limit 0.5 ng g^{-1}; Barna-Vetró et al., 1996) also indicated complete loss of OA in 5-day-old solid media (data not shown). Ochratoxin α was further degraded to an unknown compound within 7 days in solid media (Figs. 1 and 2).

4. Discussion

Mycotoxin contamination of agricultural products is a serious health hazard throughout the world. Besides attempts to eliminate mycotoxins from contaminated substrates by physical and chemical methods, the ability of microbes to degrade mycotoxins is now widely examined. Several reports describe the

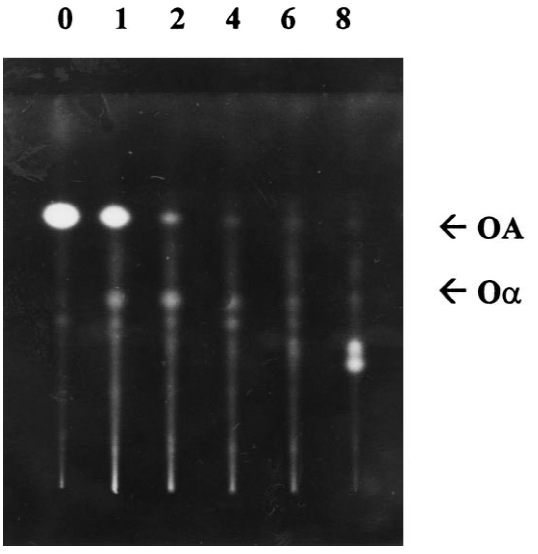

Fig. 1. Thin layer chromatography of extracts of *A. niger* CBS 120.49 cultures grown in solid media containing 2.5 μg ml^{-1} OA for 0, 1, 2, 4, 6 and 8 days at 30°C. Oα, ochratoxin α. The two strong signals in extracts of 8-day-old cultures are *A. niger* metabolites.

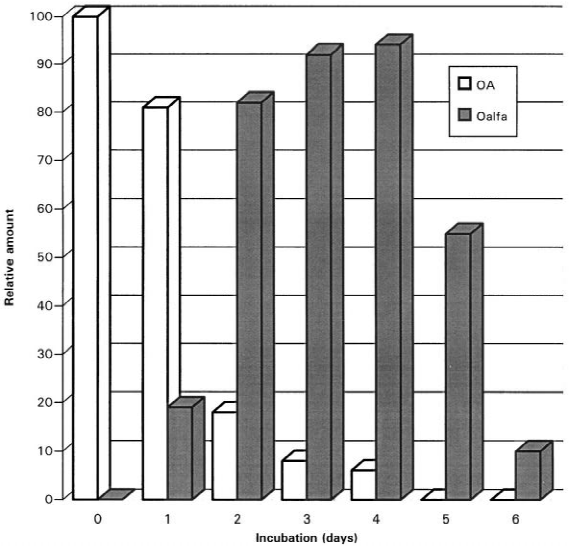

Fig. 2. Decomposition of ochratoxin A (OA) by *A. niger* strain CBS 120.49 on solid YES plates containing 2.5 μg ml^{-1} OA. The relative amounts of OA and ochratoxin α were estimated based on HPLC chromatograms. Oalfa, ochratoxin α.

OA degrading activities of the microbial flora of the mammalian gastrointestinal tract, including rumen microbes of the cow and sheep (Galtier and Alvinerie, 1976; Hult et al., 1976; Xiao et al., 1991), and microbes living mainly in the caecum and large intestine of rats (Madhyastha et al., 1992). The human intestinal microflora can also partially degrade OA (Akiyama et al., 1997). The species responsible for OA detoxification have not yet been identified, although mainly protozoa were suggested to take part in the biotransformation process in ruminants (Kiessling et al., 1984). In addition, *Butyrivibrio fibrisolvens*, a rumen bacterium, was also reported to detoxify OA to some extent (Westlake et al., 1987). Degradation of OA was observed in milk due to the action of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* species (Skrinjar et al., 1996), while two other bacteria, *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994) and *Phenylobacterium immobile* (Wegst and Lingens, 1983), were reported to also convert OA to the much less toxic ochratoxin α in liquid cultures.

We examined the OA decomposing activities of a number of *Aspergillus* strains. Both OA producers and OA nonproducing strains were tested, since producing strains were found to be able to further metabolize OA over time (Damoglou et al., 1984). None of the OA producers could significantly lower the OA content of the medium under the experimental conditions applied (data not shown). *A. fumigatus*, *A. japonicus* and *A. niger* strains were found to be able to degrade OA in liquid YES media. Although Hwang and Draughon (1994) found that *A. niger* is unable to degrade OA, an *A. niger* isolate was later reported to be able to convert OA to ochratoxin α (Xiao et al., 1996). Since *A. fumigatus* isolates themselves pose a serious health hazard due not only to their mycotoxin producing abilities (Samson et al., 1990), but also as main causative agents of different kinds of aspergilloses (Marsh et al., 1979), an *A. niger* strain was selected for further studies. *A. niger* strains are frequently used in the food industry for the production of different enzymes and organic acids (Campbell-Platt and Cook, 1989). *A. niger* is one of the few fungal species which has received the GRAS (generally regarded as safe) status from the U.S. Food and Drug Administration due to its low toxigenicity. It is worth mentioning that some isolates of both *A. fumigatus* and *A. niger* were found to produce OA in low quantities (Varga et al., 1996; Abarca et al., 1997). OA production was not observed in any of the *Aspergillus* strains which could

decompose OA in this study (Téren et al., 1996; Varga et al., 1996).

The kinetics of OA degradation of *A. niger* strain CBS 120.49 were examined in detail. This isolate could degrade OA faster in solid media than in liquid cultures (Fig. 1). The OA-degrading bacteria described so far can only be applied in substrates with high water activities such as milk (Skrinjar et al., 1996). The pathway of OA degradation in *A. niger* could be similar to that responsible for OA detoxification in *Phenylobacterium immobile* (Wegst and Lingens, 1983), which is reminiscent of the degradation of aromatic amino acids. Alternatively, a carboxypeptidase secreted by the *A. niger* strain could decompose OA to ochratoxin α and phenylalanine. Carboxypeptidase A was earlier found to be able to convert OA to ochratoxin α (Deberghes et al., 1995).

A. niger could also degrade ochratoxin α to an unknown compound. The OA-degrading microorganisms described so far can mostly convert OA to ochratoxin α, which still has limited toxicity (Harwig, 1974). An exception is the report of Galtier and Alvinerie (1976), who found that the microbial flora of animals can attack the isocoumarin ring. However, the pathway leading to the opening of the isocoumarin ring is unknown. Further biochemical studies are in progress to identify the enzyme(s) responsible for ochratoxin α decomposition.

In conclusion, an atoxigenic *A. niger* strain was found to decompose OA in both liquid and solid media. This observation is promising because it might allow the biological elimination of this mycotoxin from solid substrates such as green coffee beans and cereals. Alternatively, this fungus may provide a source of enzymes which could be used for the detoxification of OA in contaminated agricultural products. Further studies are in progress to examine the ability of this strain to degrade OA in agricultural products under different culture conditions, and to determine which enzymes take part in the detoxification process.

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