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CYCLOPIAZONIC ACID PRODUCTION BY AFLATOXIGENIC AND NON-AFLATOXIGENIC STRAINS OF ASPERGILLUS FLAVUS

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Abstract

Twenty-eight of 54 isolates of *Aspergillus flavus* grown on autoclaved agricultural commodities such as wheat, rice and corn were found to produce the mycotoxin cyclopiazonic acid. Eighteen of the *A. flavus* isolates produced aflatoxin, and fourteen isolates produced both cyclopiazonic acid and aflatoxin. A preliminary screening of some aflatoxin-contaminated corn samples revealed for the first time the natural occurrence of cyclopiazonic acid in agricultural commodities.

Introduction

Cyclopiazonid acid is a toxic indole tetramic acid which was first isolated from cultures of *Penicillium cyclopium* in 1968 (8), and subsequently found to be produced by *A. versicolor* (23) and *A. flavus* Link (7, 18). Investigations on flavutoxin, a toxic nitrogen-containing metabolite of *A. flavus* (15), subsequently revealed that flavutoxin was a metal chelate-complex of cyclopiazonic acid (7). We had detected the production of cyclopiazonic acid by several *A. flavus* isolates which also produced aflatoxin, and thus decided to survey a diverse range of *A. flavus* isolates to see if this co-occurrence was common or exceptional. We hypothesized that if cyclopiazonic acid production by aflatoxigenic strains of *A. flavus* was more common than appreciated, then this might indicate the possibility of a natural occurrence of this mycotoxin in agricultural commodities in which aflatoxins were detectable.

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Materials and methods

Growth of A. flavus *isolates on rice media for toxin production.*

Rice media was prepared by placing 75 gm polished rice and 30 ml of distilled water in each 1 L cotton stoppered Erlenmeyer flask and the flasks were placed at room temperature for 1 hr to allow for imbibition. All flasks containing media were autoclaved for 15 min, allowed to cool and a 1 ml phosphate buffered saline solution (pH 7.4) spore suspension of each isolate was seeded on the medium in one of the flasks. All flasks were incubated at 27°C for one week and agitated by hand daily.

Toxin extraction

Each culture was extracted with chloroform (2 × 300 ml). The first extraction was for 8 hr and the second extraction was overnight. The respective extracts from each culture were combined, filtered through Whatman No. 1 filter paper, and evaporated to dryness under flowing nitrogen on a steam bath. Each extract was redissolved in 5 ml chloroform and used for thin-layer chromatography.

Thin-Layer Chromatographic (TLC) Analysis

For cyclopiazonic acid analysis, 25 µl of each extract was spotted on silica gel 60 analytical thin-layer plates[1] that had been pre-treated by spraying until wet with 2% aqueous oxalic acid and dried 1 hr at 100°C. Appropriate internal and external standards were included on each plate and the plates were developed in chloroform : methylisobutylketone (4 : 1 v/v) (29). The plates were removed from the tank, dried at room temperature, sprayed with 1% p-dimethyla-

minobenzaldehyde in 95% ethanol, dried with warm air and placed in a tank containing HCl vapor for 10 min to allow for color development. Cyclopiazonic acid had an Rf value of 0.65 and was lavender to purple in color. Alternatively, the plates may be sprayed with 1% ethanolic solution of $FeCl_3$ resulting in a reddish-orange color of the cyclopiazonic acid.

Cyclopiazonic acid analysis was also conducted using silica gel 60 plates without oxalic acid impregnation. These plates were developed in toluene : ethyl acetate : formic acid (5 : 4 : 1 v/v/v) solvent system resulting in a greatly reduced mobility of cyclopiazonic acid which can be visualized with either of the above color reagents.

For aflatoxin analysis, 25 μl of each extract as well as appropriate internal and external standards was spotted on silica gel 60 analytical thin layer plates. The plates were developed with chloroform : methanol : formic acid (97 : 2 : 1 v/v/v), dried and viewed under long wavelength UV light. Confirmation of aflatoxins was conducted by spraying the plates with 25% aqueous sulphuric acid and viewing the plates again under long wavelength UV light for the color change of the aflatoxins from fluorescent blue and green to a fluorescent yellow.

Production of cyclopiazonic acid on wheat and corn.

Cracked red wheat medium and corn medium were prepared in 1 L Erlenmeyer cotton stoppered flasks in the same manner as was the rice media. Each of four isolates, selected for their ability to produce cyclopiazonic acid on rice media, were inoculated in two flasks of the wheat medium. The four isolates selected were: NADC 36% ration, NRRL 1290, NRRL 6388 and NRRL 3251. Isolate NRRL 6388 was inoculated in two flasks containing corn medium. All cultures were incubated with daily agitation at 27°C for two weeks. The cultures were extracted and examined for cyclopiazonic acid production in the same manner as were the rice cultures.

Isolation of cyclopiazonic acid from naturally contaminated corn.

A 50 gm portion of a dry ground sample was blended with 100 ml of chloroform for 2 min in a Waring Blender. The slurry formed was poured into a 500 ml flask, a further 100 ml of chloroform added, and the slurry stirred for 3 hr. The slurry was filtered through a Whatman No. 1 filter paper into a 500 ml separatory funnel, and 200 ml of 1N potassium bicarbonate solution added. The funnel was shaken and the two layers were allowed to separate. The lower layer was discarded, and the remaining aqueous phase was then acidified by careful addition of 40 ml of 5N hydrochloric acid with gentle swirling until all CO_2 evolution had ceased. The pH was checked to ensure that it was in the range 1 to 2, and the aqueous phase extracted with chloroform (3 × 100 ml). The combined chloroform extracts were dried over anhydrous sodium sulphate, filtered, and evaporated under vacuum. The resulting residue was dissolved in 1 ml of chloroform, and a 100 μl aliquot examined by TLC (see above) for the presence of cyclopiazonic acid.

Isolation of cyclopiazonic acid from the corn extract, for confimation by mass spectroscopy, was achieved by preparative TLC as follows. A 20 × 20 cm precoat silica gel 60 plate (0.25 mm thick silica gel layer) was treated with 2% oxalic acid solution (see above). The total 1 ml concentrated extract (above) was applied as a horizontal streak 2 cm from the base of the plate, and the plate developed in chloroform : 2-pentanone (4 : 1 v/v). The position of the cyclopiazonic acid band was detected by spraying a 1 cm wide vertical strip of the plate with p-dimethylaminobenzaldehyde reagent; the rest of the plate was protected from the reagent and HCl vapor exposure by sealing with aluminium foil. Exposure to HCl vapor revealed the position of the cyclopiazonic acid as a purple band; examination of the whole plate under long wavelength UV light helped to reveal the exact position of the band. The unsprayed cyclopiazonic acid band thus identified was scraped from the TLC plate, and the recovered silica gel crushed to a fine powder and eluted in a small glass column (plugged at the lower end with glass wool) with 5 ml of chloroform : methanol (4 : 1 v/v). The eluted solvent was evaporated to yield cyclopiazonic acid of sufficient purity for mass spectral characterization.

Results and discussion

Fifty-four *A. flavus* isolates from a variety of sources were cultured in the laboratory, and the cultures extracted with chloroform and the chloroform extracts checked by thin layer chromatography for the presence of cyclopiazonic acid and aflatoxins (Table I).

A major portion of the isolates were obtained from investigations of moldy corn in Iowa (24, 25). Of the others, 22 were known to be isolated from animal tissues,

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Table 1. Occurrence of Cyclopiazonic Acid and Aflatoxins in *A. flavus* Isolates.

Isolate Identification	Source	Cyclopiazonic Acid	Aflatoxins
MC-16	Corn-NADC	+	+
MC-17	Corn-NADC	+	
MC-21	Corn-NADC	Slight	+
MC-22	Corn-NADC	+	+
MC-33	Corn-NADC		
MC-38	Corn-NADC	+	+
MC-43	Corn-NADC		
MC-48	Corn-NADC	+	+
MC-63	Corn-NADC		
MC-77	Corn-NADC	+	+
MC-83	Corn-NADC	Slight	
MC-92	Corn-NADC	+	+
MC-115	Corn-NADC	+	
MC-171	Corn-NADC		
MC-172	Corn-NADC		
Corn-Tif	Corn		
Nelson Corn	Corn		
NRRL 5519	White corn	Slight	
OK-4	Corn-High moisture		
OK-7	Corn-High moisture	+	
Barley-Tif	Barley		+
NRRL 2999*	Ugandan ground nuts		+
NADC 18% ration	Guinea pig ration	+	
NADC 36% ration	Guinea pig ration	+	
Jukes feed	Turkey ration	Slight	
Jackson pellets	Mixed feed	Slight	
NRRL 3251	Walnuts	+	+
Pin-2-1F	Turkey house litter		
Pin-3-7A	Turkey house litter		
NRRL 1290	Human throat	+	+
NRRL 488	Human		
NRRL 1276	Human, ear		
NRRL 487	Human, duodenum content		
NRRL 6381	Human		+
NRRL 6382	Human sphenoid sinus	+	+
NRRL 6384	Human-Farmer's lung	Slight	
NRRL 6386	Human		
NRRL 6387	Human	+	+
Buginas	Human		+
Johnson	Human		
Martinez	Human	Slight	+
Hangen	Human		
Berhow	Human	+	
NRRL 498	Brazil "Macuco" bird	+	+
NRRL 6383	Marsh hen, air sac		
NRRL 6385	Turkey-lung		
NRRL 6388	Penguin	+	
CDC-B-2101	Parrot	+	
73-P-2202	Cat		
63-IV-78	Sheep adrenal gland-NADC	+	+
63-IV-75	Lamb fetal stomach-NADC	Slight	
CRC-76	Unknown		
M93-16HEW	Unknown		+
CDC-1	NCDC Teaching Collection		
	TOTALS	28	18

**A. parasiticus.*

including at least nine from human lung tissues.

Of the 54 *A. flavus* isolates, 28 were found to produce cyclopiazonic acid under the culture conditions utilized. This relatively high proportion of cyclopiazonic acid producers indicates a much higher frequency of ability of *A. flavus* to produce this mycotoxin than previously recognized. These findings of our present investigation, together with a recent report that cyclopiazonic acid is produced by a number of *Penicillium species* isolated from various meat substrates, especially sausage (17), indicates that cyclopiazonic acid may be more widespread as a fungal metabolite in feeds and foods than is known at the present time.

Eighteen of the *A. flavus* isolates produced aflatoxin, and it is noteworthy that although aflatoxin-producing ability did not correlate with cyclopiazonic acid-producing ability, a high percentage (77%) of aflatoxin producers also produced cyclopiazonic acid. Thus, 14 isolates produced both mycotoxins, whereas 4 isolates produced aflatoxin only and 14 isolates produced cyclopiazonic acid alone. The possible importance of synergistic and additive interactions of aflatoxin and cyclopiazonic acid, when they co-occur, is being (further) investigated.

The *A. flavus* isolates tested produced the mycotoxins on typical agricultural commodities: wheat, rice, and corn. Thus it appeared quite possible that a screening of natural aflatoxin-contaminated agricultural commodities might reveal instances of cyclopiazonic acid contamination. For a preliminary check on this, we chose to examine some corn samples which had been found to have a relatively high aflatoxin content due to natural contamination.

There is no published method for the quantitative analysis of cyclopiazonic acid in naturally contaminated agricultural substrates. However, we found that the acidic nature of the toxin enabled qualitative detection to be achieved in samples of naturally contaminated corn. Partitioning procedures based on the acidic nature of cyclopiazonic acid have been used for its qualitative detection and isolation from fungal cultures (8, 9, 18). A chloroform extract of the ground substrate was extracted with potassium bicarbonate solution, which converted the toxin into its water-soluble potassium salt form. Subsequent acidification and partitioning back into chloroform gave a relatively clean extract, in which cyclopiazonic acid, when present, was detected by TLC. Although the % recovery of cyclopiazonic acid by the method was not determined, grain samples 'spiked' with crude cyclopiazonic acid isolated from fungal cultures yielded good recoveries when extracted in the above manner.

With the above methods, we obtained evidence for the first time reported, of the natural occurrence of this toxin in corn, both stored and directly from the field (Table 2). The presence of cyclopiazonic acid in one of the samples (Georgia corn NADC 4–77) was confirmed by isolation

Table 2. Cyclopiazonic acid analysis of corn samples from field (F) and storage (S).

Sample Source & Identification	Aflatoxin Content	Cyclopiazonic acid
ISU - VDL - Cam. Elev. Iowa (S) . . .	49 ppb B_1	+, TLC
NADC - 3-77 - Iowa (F).	2000 ppb B_1	+, TLC
NADC - 4-77 - Georgia (F)	605 ppb B_1	+*, TLC, confirmed MS
NADC - 5-77 - Georgia (F)	194 ppb B_1	+, TLC
NADC - 6-77 - Georgia (F)	600 ppb B_1	+, TLC
NADC - 7-77 - Georgia (F)	1036 ppb B_1	+, TLC

*estimation of cyclopiazonic acid content: 10 ppm.

of the toxin by preparative TLC, followed by mass spectral characterization. The molecular ion in the mass spectrum at m/e 336, and associated peaks at m/e 196, 182, 181, 155, 154 (5, 8) together with TLC behavior (mobility and Rf value in two systems), color reactions, and acidic nature served to unequivocally identify cyclopiazonic acid.

Apart from cyclopiazonic acid, a number of tetramic acids have been isolated to date from microorganisms. The most well known of these is tenuazonic acid, which has been isolated from *Alternaria species* (4, 14, 21, 22, 27), *Piricularia oryzae* (31), an *Aspergillus species* (13), and from *Phoma sorghina* (30). Tenuazonic acid has significant biological activity: it is a mycotoxin, phytotoxin, an antibiotic, and it also has antitumor properties (13). Other fungal tetramic acids include erythroskyrine from *Penicillium islandicum* (10, 28), and equisetin, an antibiotic from *Fusarium equiseti* (2). Also, it appears that phomopsin A, the main toxic metabolite of *Phomopsis leptostromiformis* responsible for lupinosis disease of sheep, may be a tetramic acid (3). Some antibiotic tetramic acids produced by other microorganisms include magnesidin from *Pseudomonas magnesiorubra* (16), and the following from *Streptomyces spp.*: ikarugamycin (11, 12), streptolydigin (6, 26), tirandamycin (19, 20) and antibiotic K16 (1).

It is important to note for both biological and chemical investigations that the tetramic acids appear to occur naturally as metal chelate complexes. Thus, we found cyclopiazonic acid to exist as a metal chelate complex in crude extracts which had not been treated with strong acid (7). Similarly, tenuzonic acid was isolated from *Phoma sorghina* cultures as magnesium and calcium chelate complexes (30), and from *Piricularia oryzae* cultures as (partly) an iron III chelate complex (31). The tetramic acid magnesidin was isolated as a magnesium complex (16). Treatment of solutions of the metal complexes with dilute mineral acid and partitioning into solvent is sufficient to decompose the complex and release the parent compound: in this way, cyclopiazonic acid is freed from cations (7) as is tenuazonic acid (30). Since the isolations of most of the tetramic acids enumerated above involved a similar acid treatment/solvent partition step at some stage in their purification, it is likely that the metal complexes are the natural form of the tetramic acids in cultures and substrates where they are produced. Apparently, the acidic and enolic nature of the tetramic acid nucleus (32) and the spatial arrangement of the enolic oxygens is favorable for complex formation. Thus, the tetramic acids readily form stable complexes with iron and copper: the red or red-orange ferric complexes and greenish cupric complexes provide useful presumptive evidence for the presence of a tetramic acid nucleus. Complex formation has been utilized for purification purposes: tenuazonic acid has been purified via its copper II complex (22, 27, 30).

The metal chelate complex forms of the tetramic acids can present difficulties in spectroscopic analysis. Thus flavutoxin (15), a metal chelate complex of cyclopiazonic acid (7), only gave a weak mass spectrum, which required strong heating of the mass spectrometer probe. In this case, a molecular ion was not observed: instead, weak peaks occurred at m/e 155, 154 and 127. In contrast, cyclopiazonic acid itself gives an intense mass spectrum under conditions of only gentle heating: it shows a strong molecular ion at m/e 336, with an intense base peak at m/e 182. Similar behavior was reported for tenuazonic acid: the metal complex gave only a weak fragment ion at m/e 141, c.f. the parent acid which gives a molecular ion at m/e 197, and a base peak at m/e 141 (30, 31). Again, the poor solubility of flavutoxin in the usual nuclear magnetic resonance spectral solvents resulted in only a very weak low intensity NMR spectrum (7, 15). This problem was aggravated by the presence of the strongly diamagnetic cations of the complex which interferred with most of the signals from the rest of the covalent molecule (7).

Further evidence of the metal-chelating ability of cyclopiazonic acid is seen in its behavior on TLC. The acid migrates slowly with low Rf value, and extensive tailing occurs, on TLC on silica gel G with various solvent systems. This behavior is clearly a result of metal chelation with the toxin: oxalic acid (or tartaric acid) impregnation of the silica gel causes cyclopiazonic acid to travel with much higher Rf and as a compact spot or zone (18, 29). Presumably, the oxalic acid (or tartaric acid) effectively competes with cyclopiazonic acid for cation sites in the silica gel matrix. The dramatic difference in mobility of cyclopiazonic acid in the two TLC systems (i.e. silical gel, and silica gel impregnated with oxalic acid), plus intense color reactions (purple with Ehrlich's reagent indicating an indole nucleus, and red with ferric chloride, indicating a tetramic acid nucleus) form the basis of a very reliable diagnostic test for cyclopiazonic acid, used in the present investigation.

The ability of cyclopiazonic acid and other tetramic acids to complex with metal cations may be an important factor in their *in vivo* toxicity and assay.

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