Toxic Metabolite Produced by *Aspergillus wentii*

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Mycelial extracts of an *Aspergillus wentii* strain grown on yeast-extract sucrose medium and initially isolated from country-cured ham were highly toxic when inoculated into chicken embryos or fed to mice. Moldy corn and rice were less toxic when fed to mice. Water extracts of moldy corn or rice or culture filtrates from yeast-extract sucrose medium were not toxic. Purification by thin-layer chromatography followed by crystallization yielded orange-red crystals that showed high toxicity and had a melting point of 285 to 286 C. Chloroform solutions of the crystals had absorption maxima at 270, 295, and 452 nm. The smallest amount of this component necessary to have zero hatchability of fertile eggs was 50 μg/egg.

Toxicity of *Aspergillus wentii* to animals was first reported by Rabie et al. (5). They found that strains of *A. wentii* grown on corn caused toxic symptoms and death in ducklings, chickens, rabbits, and sheep. The most consistent lesions were found in the liver (4). Higher toxicity was reported for one strain at incubation temperatures of 20 to 25 C than at 30 C, and higher toxicity was reported for another strain at 25 C, compared with 20 and 30 C. Ether extracts of *A. wentii* isolated from corn and grown on rice were toxic to mice but not ducklings when administered intraperitoneally (6). Semeniuik et al. (9) found several strains of *A. wentii* to be lethal for mice when grown on wheat. In a survey reported by Scott (8) of toxigenic fungi isolated from cereal and legume products, none of five strains of *A. wentii* was toxic to ducklings. Recently, Chassis et al. (Proc. Ass. Southern Agr. Workers, Jacksonville, 1971.) reported that corn infected with *A. wentii* isolated from country-cured ham was toxic when fed to mice.

The present investigation was undertaken to further study the toxicity of *A. wentii* isolated from country-cured ham and to purify and characterize the principal toxin(s) produced by this strain.

**MATERIALS AND METHODS**

*A. wentii* M-108 previously isolated from a country-cured ham by Leistner and Ayres (3) was transferred into Czapek Dox agar (CDA). For preparation of moldy corn and rice, 1 kg of substrate was autoclaved for 1 h in a 2.8-liter Fernbach flask. After the media cooled, 10⁶ spores harvested from CDA and 300 ml of sterile distilled water were added. The inoculated substrates were then incubated at 27 C for 2 months; after incubation, the moldy substrates were autoclaved for 1 h. In a second experiment, 10⁶ spores were inoculated into 100 ml of sterilized yeast-extract sucrose (YES) medium (2% yeast extract, 20% sucrose) in 500-ml Erlenmeyer flasks, and the substrate was incubated at 27 C for 1 month. After autoclaving, mycelia were separated from the culture filtrate and air-dried.

Chicken embryo inoculation (10) and mouse feeding were used to assay the toxicity of moldy substrates. For chicken embryo assays, dry moldy corn, rice, and mycelia were ground in a no. 3 Wiley mill. Fifty grams of mycelial powder from YES medium was extracted using chloroform in a Soxhlet extractor. Also, 50 g of moldy corn or rice was similarly extracted. After 16 h of extraction, the extract was concentrated in a flash evaporator and diluted to 5 ml with chloroform. Various dilutions were also made for testing the level of toxicity. Groups of 20 fertile White Leghorn eggs were inoculated before incubation with 0.02 ml of water or chloroform extract or with culture filtrate from YES medium. Control groups of eggs were injected with chloroform or water, with chloroform or water extract of uninoculated corn and rice, or with sterilized uninoculated YES medium. For mouse feeding tests, ground moldy rice and corn and dry mycelia were mixed with ground Purina mouse chow (Ralston Purina Co., St. Louis) in different ratios. Chloroform extracts of mycelia and culture filtrates of YES medium were also mixed with mouse chow in different ratios. These mixtures were placed under vacuum to remove chloroform. Ten 2-week-old white Swiss mice were used in each treatment. Control groups were fed mixtures of uninoculated rice and corn with mouse chow in corresponding ratios. Water and feed were available ad libitum. The assay lasted 5 months. The criteria for positive response were weakness, recumbence, and death.

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The chloroform extract of mycelia was separated by thin-layer chromatography (TLC) on Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) with chloroform-acetone 95:5 (vol/vol) as the developing solvent. To obtain more material for assay of toxicity, 0.2 ml of chloroform extract was applied as a line on a TLC plate. After developing, the plate was divided into 13 bands according to color and fluorescence under ultraviolet (UV) light. Each band was removed and extracted with chloroform and acetone mixture. The bands with high $R_f$ values required more chloroform in the mixture for extraction. The extracts from five plates were concentrated to 1 ml and injected into 20 fertile eggs (0.02 ml/egg) for each assay of toxicity; duplicate tests were made.

RESULTS AND DISCUSSION

Of the 13 bands observed on TLC plates, band 6 ($R_f$ 0.57), which proved to be the most toxic, was collected from many plates and extracted with a mixture of chloroform and acetone 50:50 (vol/vol). Purity of this compound was checked with other solvent systems (chloroform-acetone, 50:50; toluene-ethyl acetate-formic acid, 6:4:1; chloroform-ethanol, 95:5; benzene-ether, 5:2). When these plates were charred with sulfuric acid solution, only a single spot appeared.

The extracted no. 6 bands were combined and concentrated, and the crystals formed were collected and dried under vacuum. The melting point of the crystals was determined by using a melting point apparatus. A Beckman DBG spectrophotometer was used to determine the absorption spectrum in the UV and visible ranges. Toxicity of this purified material was assayed by using the chicken embryo test.

Table 1 shows the toxicity of different extracts to the chicken embryos. The culture filtrate of YES medium and water extracts of moldy corn and rice were not toxic to chicken embryos. Chloroform extracts of moldy corn were toxic to chicken embryos when introduced at full strength (0.02 ml) and slightly toxic at one-tenth that of the original concentrated extract. The chloroform extracts of moldy rice were moderately toxic, and no toxicity was observed at low concentration. Chloroform extracts of mycelia also were highly toxic. The extract from 10 mg of mycelia was the minimum amount necessary to obtain zero hatchability in 20 eggs.

As shown in Table 2, moldy corn mixed with mouse chow (1:1) killed mice in 58 to 70 days, whereas a low ratio of moldy corn to chow (1:9) did not kill but only retarded growth. Moldy rice at 1:1 and 1:9 ratios did not kill mice. Mycelia from YES medium was the most toxic substrate. A mycelia-mouse chow mixture of 1:9 killed all 10 mice within 4 to 6 days. On the other hand, culture filtrates were not toxic to mice. Mixtures of culture filtrate and mouse chow did not kill mice or retard their growth.

Chloroform extracts of mycelia were highly toxic to mice. Mice were killed within 3 to 16 days when fed mixtures of chloroform extract and mouse chow at a ratio of 1:9 or at 1:250. There was no particular symptom before mice died except that their growth was retarded. They became weak, recumbent, and moribund.

**Table 1. Toxicity of different extracts to the chicken embryo**

| Treatment                                      | Hatchability (%) |
|------------------------------------------------|------------------|
| Control, nontreated                            | 100*             |
| Sterilized distilled water                     | 95               |
| Chloroform                                     | 100              |
| Sterilized uninoculated YES medium             | 98               |
| Sterilized culture filtrate of YES medium      | 95               |
| Water extract of moldy corn                    | 0                |
| Water extract of moldy rice                    | 0                |
| Chloroform extract of moldy corn (1/10)        | 85               |
| Chloroform extract of moldy rice               | 60               |
| Diluted chloroform extract of moldy rice (1/10)| 93               |
| Chloroform extract of 200 mg of mycelia        | 0                |
| Chloroform extract of 50 mg of mycelia         | 0                |
| Chloroform extract of 10 mg of mycelia         | 0                |
| Chloroform extract of 9 mg of mycelia          | 15               |
| Chloroform extract of 2 mg of mycelia          | 70               |

*Average of two replications; each replication consists of 20 eggs.

**Table 2. Toxicity of moldy corn, rice, and mycelia of A. wentii when fed to mice**

| Feed                                      | No. died | Days to death |
|-------------------------------------------|----------|---------------|
| Moldy corn-chow (1:1)                    | 10       | 58-70         |
| Moldy corn-chow (1:9)                    | 0        | 0             |
| Moldy rice-chow (1:1)                    | 0        | 0             |
| Moldy rice-chow (1:9)                    | 0        | 0             |
| Mycelia-chow (1:9)                       | 10       | 4-6           |
| Chloroform extract of mycelia-chow (1:5)  | 10       | 3-5           |
| Chloroform extract of mycelia-chow (1:25) | 10       | 4-5           |
| Chloroform extract of mycelia-chow (1:50) | 10       | 6-7           |
| Chloroform extract of mycelia-chow (1:250)| 10       | 14-16         |
| Culture filtrate-chow (1:3)              | 0        | 0             |
| Uninoculated corn-chow (1:1)             | 0        | 0             |
| Uninoculated corn-chow (1:9)             | 0        | 0             |
| Uninoculated rice-chow (1:1)             | 0        | 0             |
| Uninoculated rice-chow (1:9)             | 0        | 0             |
| Whole chow                               | 0        | 0             |
It is apparent that toxin(s) of this fungus is intracellular.

Mixtures of uninoculated corn and mouse chow and uninoculated rice and chow were not toxic to mice.

Since the chloroform extract of mycelia from YES medium showed the highest toxicity, isolation and purification of toxin(s) were attempted from this extract. The chloroform extract of mycelia was a dark-brown viscous liquid. Table 3 shows the relative toxicities of 13 bands from the TLC plates on chicken embryo hatchability, and band 6 apparently was the most toxic. Other bands such as 1, 2, 4, 7, 8, and 11 also show some toxicity. In this study, we only worked on band 6. The toxin appeared as a single orange-red band (R, 0.57) on TLC plates. The toxin was soluble in chloroform, acetone, benzene, and ethyl acetate, insoluble in ethanol, and did not fluoresce under UV light. Crystallization from the chloroform-acetone 50:50 (vol/vol) yielded orange-red needle-shaped crystals having a melting point at 285 to 286 C. The UV and visible spectra showed absorption maxima in chloroform at 270, 295, and 452 nm. The minimum amount of this toxin necessary to have zero hatchability was 50 μg/egg (Table 4).

**Table 3. Toxicity of different components of chloroform extract* of mycelia to the chicken embryo**

| Band no. | Hatchability (%) |
|----------|------------------|
| 1        | 40               |
| 2        | 33               |
| 3        | 98               |
| 4        | 50               |
| 5        | 83               |
| 6        | 0                |
| 7        | 43               |
| 8        | 35               |
| 9        | 100              |
| 10       | 70               |
| 11       | 55               |
| 12       | 95               |
| 13       | 70               |
| Control, nontreated | 100          |
| Chloroform | 98        |

*Extract from five TLC plates; 0.2 ml of concentrated extract was applied on each plate.

*Average of two replications; each replication consists of 20 eggs.

**Table 4. Toxicity of different amounts of pure compound from band no. 6 to the chicken embryo**

| Amount (μg/egg) | Hatchability (%) |
|-----------------|------------------|
| 100             | 0*               |
| 75              | 0                |
| 50              | 0                |
| 45              | 23               |
| 40              | 35               |
| 30              | 70               |
| 10              | 100              |
| Control, nontreated | 98        |
| Chloroform      | 100              |

*Average of two replications; each replication consists of 20 eggs.

Chromatographic study showed this metabolite to differ from aflatoxins (B₁, B₂, G₁, G₂) and verruculogen (1, 2, 7).

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