Production of Ochratoxin A by *Aspergillus ochraceus* Isolated in Japan from Moldy Rice

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A simple thin-layer chromatography-fluorodensitometric method for quantitative analysis of ochratoxin A was developed. This method proved to be of use in investigating the production of the toxin and the nutritional factors affecting the toxin production by two strains of *Aspergillus ochraceus* isolated from moldy rice in Japan. These fungi produced large amounts of ochratoxin A in a nutrient solution containing 1% L-phenylalanine and 2% yeast extract.

Ochratoxin A, first described by van der Merwe et al. (7, 8), is a toxic metabolite of certain strains of *Aspergillus ochraceus* Willh. isolated from African cereals and legume products.

We isolated 457 strains of *A. ochraceus* in this country from moldy rice during a period from 1968 to 1969. Fifty-eight of these strains were randomly subjected to toxicity tests performed in mice and chick embryos, and two strains were found to be strongly toxigenic and to produce ochratoxin (MR 31-1 and MTR 26-1).

The spot of ochratoxin A developed by thin-layer chromatography (TLC) can easily be located by its strong fluorescence under ultraviolet light. Steyn and van der Merwe (5) applied TLC to the NaHCO₃-soluble portion of a CHCl₃-MeOH (1:1) extract of moldy materials and estimated the amounts of ochratoxin A by the sizes of the fluorescent spots as compared to spots of standard solutions of ochratoxin A which were co-chromatographed. Scott and Hand (4) also described a TLC method for estimating ochratoxin A contents. By using a similar method, Ferreira (2) and Davis et al. (1) studied the effects of various carbon and nitrogen sources added to a semisynthetic medium on ochratoxin A production by *A. ochraceus*.

The present paper deals with a simple fluorodensitometric method coupled with TLC for quantitative analyses for ochratoxin A. The toxin production by the two strains under different nutritional conditions as analyzed by the method is also described.

**MATERIALS AND METHODS**

**Organisms.** *A. ochraceus* strains MR 31-1 and MTR 26-1 were isolated in this laboratory from moldy rice collected in Chiba and Miyagi Prefectures during a period from 1968 to 1969. The strains were maintained on malt extract agar and on Czapek agar containing 20% sucrose.

**Cultures.** To examine the toxin production on rice, 50 g of rice and 25 ml of water were dispensed into each of four Erlemeyer flasks, which were autoclaved for 20 min at 120 °C and then seeded with each strain. The flasks were incubated in the dark for 9 days at 27 °C with occasional shaking.

To examine the effects of different nitrogen sources on toxin production, 2% yeast extract, 2% polypeptide, 2% malt extract, or 2% yeast extract plus 1% L-phenylalanine was added to a basal medium with a formula similar to that described by Ferreira (2). One liter of the basal medium contained: 1.0 g of KH₂PO₄, 0.5 g of KCl, 0.5 g of MgSO₄·7H₂O, 24.4 mg of FeCl₃·6H₂O, 21.99 mg of ZnSO₄·7H₂O, 10.98 mg of MnSO₄·5H₂O, 3.93 mg of CuSO₄·5H₂O, 2.52 mg of (NH₄)₂MnO₄·4H₂O, and 40 g of sucrose.

Incubation was carried out in a stationary manner in the dark for 16 days at 27 °C.

**Extraction of the toxin and preparation of samples for TLC.** The mycelia grown on rice were harvested and extracted repeatedly with ethyl acetate, and the solvent was then evaporated in vacuo. The mycelia grown in a liquid medium were separated from the mother liquid. The mycelia were dried and extracted quantitatively with ethyl acetate. The culture filtrate was adjusted to pH 3.0 and extracted with CHCl₃. After the solvent had been removed in vacuo, each extract was dissolved in CHCl₃ at an appropriate concentration, and 2- to 10-µl portions were spotted on a TLC plate with a microsyringe.

**Ochratoxin A.** *A. ochraceus* MR 31-1 was grown in Davis medium (1), and the culture was extracted with ethyl acetate. The NaHCO₃-soluble part of the extract was subjected to silica gel column chromatography. Ochratoxin A in the eluted fraction was recrystallized from benzene. The preparation proved to possess chemical and physical properties identical to those reported by van der Merwe et al. (7, 8).

**Procedures for running TLC.** The crystalline ochratoxin A, dissolved in CHCl₃ at a concentration of
0.656 mg/ml, was spotted on a TLC plate of silica gel (Merck) in 2-, 4-, 6-, 8-, and 10-μl amounts, and 2-μl amounts of its two-, four-, and eightfold dilutions were also spotted. Each sample solution was spotted in the same way. The plates were developed with benzene-methanol-ethyl acetate (15:3:1) and dried. (The solvent system was recommended by S. Natori of the National Institute of Hygienic Sciences, Tokyo. The RF values for ochratoxins B and C were 0.14 and 0.74, respectively.) The greenish-blue fluorescent spot (RF = 0.31) of ochratoxin A was located under ultraviolet light.

**Densitometric analysis.** The developed TLC plate was placed on the stage of a fluorodensitometer (Ozumor SD-91; Asuka Kogyo Co., Tokyo) at such a position that the spots of ochratoxin A would be in the light path. The plate was scanned at a wavelength of 333 nm for excitation to read the intensity of fluorescence of each spot as recorded on a chart by the integrator attached to the densitometer.

**FIG. 1. Relation of fluorodensitometric readings in response to the concentrations of ochratoxin A.**

**TABLE 1. Ochratoxin A production by the two strains of Aspergillus ochraceus on rice**

| Determination                              | Strain  |
|--------------------------------------------|---------|
|                                            | MR 31-1 | MTR 26-1 |
| Residual solid from ethyl acetate extract  | mg       | mg       |
| Ochratoxin A                               | 2.70a    | 1.15a    |
| Ochratoxin A per gram of ethyl acetate     | 135      | 40.3     |
| extract solid                              | 50       | 35       |

* Values expressed in grams.

**TABLE 2. Ochratoxin A production by the two strains of Aspergillus ochraceus in a semisynthetic medium containing 2% yeast extract**

| Determination                              | Strain  |
|--------------------------------------------|---------|
|                                            | MR 31-1 | MTR 26-1 |
| Dry weight of mycelium                     | mg       | mg       |
| Residual solid from CHCl₃ extract of mycelium | 20.2b  | 25.4b   |
| Ochratoxin A from mycelium                | 366      | 585      |
| Ochratoxin A from culture filtrate         | 30       | 10       |
|                                            | 240      | 190      |

* Initial pH of the medium was 5.9 for both MR 31-1 and MTR 26-1. Final pH was 6.8 for MR 31-1 and 6.2 for MTR 26-1. All values show the yield per liter.

b Expressed as grams.

**TABLE 3. Ochratoxin A production by Aspergillus ochraceus MR 31-1 in response to the different nitrogen sources**

| Determination                              | Growth factor added |
|--------------------------------------------|---------------------|
|                                            | 2% Yeast extract    | 2% Polypeptide      | 2% Malt extract   | 2% Yeast extract +1% L-phenylalanine |
|                                            | mg                  | mg                  | mg                | mg                             |
| Dry weight of mycelium                     | 19b                 | 19b                 | 4b                | 25.4b                          |
| Residual solid from CHCl₃ extract of mycelium | 345     | 279                | 115               | 484                           |
| Ochratoxin A from mycelium                | 32                  | 32                 | Trace             | 44                            |
| Ochratoxin A from culture filtrate         | 240                 | 384                | Trace             | 608                           |

* Initial pH of the medium was: with 2% yeast extract, 5.9; with 2% polypeptide, 6.0; with 2% malt extract, 4.8; with 2% yeast extract plus 1% L-phenylalanine, 6.2. Final pH of the medium was: with 2% yeast extract, 7.6; with 2% polypeptide, 7.8; with 2% malt extract, 4.8; with 2% yeast extract plus 1% phenylalanine, 7.5. All values show yield per liter.

b Expressed as grams.

**RESULTS AND DISCUSSION**

Densitometric readings in response to quantities of ochratoxin A on a TLC plate are illustrated in Fig. 1. A linear correlation was observed between 0 and 5 μg of the toxin. Since the smallest quantity of the toxin that the densitometer detected was approximately 0.17 μg per spot, the amounts of the toxin to be spotted on a TLC plate should be in a range between 0.17 and 5 μg. The reading did not change after the plate.
was allowed to stand for 1, 2, or 3 hr or even for 4 days in the dark at room temperature.

The amounts of ochratoxin A produced on rice by the two toxigenic strains were determined by the present method (Table 1). Strain MR 31-1 produced the toxin in a quantity approximately three times as great as that produced by strain MTR 26-1. The amounts of the toxin per gram of residual solid prepared from ethyl acetate extract were calculated to be 50 and 35 mg, respectively.

The results of similar experiments with cultures in semisynthetic liquid media are shown in Table 2. In liquid media, strain MR 31-1 also produced a larger amount of ochratoxin A than did strain MTR 26-1.

Ferreira (2) obtained a maximal yield of ochratoxin A, 100 mg/liter, when a basal medium containing 3% sucrose was supplemented with 1% glutamic acid as a nitrogen source. The addition of ammonium acetate, corn-steep liquor, or Casamino Acids reduced the yield in submerged culture. Phenylalanine might appear to increase the yield of the toxin as ochratoxins contain this amino acid as a structural component. His experiments, however, showed that the production was not affected by the addition of phenylalanine as a sole source of nitrogen. Davis et al. (1) studied the effects of various carbon and nitrogen sources and found that the addition of 4% sucrose and 2% yeast extract enhanced the production of ochratoxin in a stationary culture, attaining a yield of 290 mg/liter.

From the results shown in Table 3, it is apparent that polypeptone has as great an effect on toxin production as yeast extract; however, no effect of malt extract was observed. The addition of 1% L-phenylalanine markedly increased the yield of the toxin. Up to 600 mg of ochratoxin A was produced by the fungi in 1 liter of a medium containing 2% yeast extract and 1% L-phenylalanine.

Thus, it seems very plausible that L-phenylalana-