

Medium-Scale Production and Purification of Ochratoxin A, a Metabolite of *Aspergillus ochraceus*

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The preparation of crystalline ochratoxin A from *Aspergillus ochraceus* nutrient solution is described. Methods are adaptable to large-scale fermentations.

Several microbiologists have studied factors which influence the production of ochratoxin A by *Aspergillus ochraceus* Wilhelm (2, 4, 5, 11). However, methods reported for purification of ochratoxin A from small quantities of fermented media are unsuited to larger scale fermentations. Because of interest in its biochemical effects (3, 6, 7-9, 10; P. M. Scott and W. van Walbeek, Abstr., Fifth Great Lakes Regional Meetings, Amer. Chem. Soc., 1971), procedures have been developed for medium- and large-scale production and purification of ochratoxin A.

A. ochraceus NRRL 3174 was used throughout this investigation. Cultures were maintained at 5 C on slants of Czapek agar with 20% sucrose and 0.7% yeast extract.

Pyrex carboys (5-gal) containing 4 liters of 4% sucrose and 2% yeast extract nutrient solution were stoppered with cotton plugs and autoclaved 25 min at 121 C. Media were inoculated with spores from 1-week-old cultures of *A. ochraceus* and incubated for 7 days at 25 C as stationary cultures. Carboys were placed on their sides to give a maximum surface area to volume ratio. Seven experiments were conducted with four carboys (16 liters of medium) per experiment.

The isolation procedure is given in Fig. 1. Confirmatory tests for ochratoxin A have been previously described (2, 9). Identity of the final product was also confirmed by low-resolution mass spectra obtained with the solid-sample inlet of a Du Pont 21-490 mass spectrometer. Final yield of ochratoxin A was determined gravimetrically. Analytical procedures were carried out under reduced light at room temperature.

After incubation, cultures were filtered

through cheesecloth, and the filtrate was passed slowly through a column of 200- to 400-mesh Dowex 1-X8 resin (formate form). The column was prepared by placing 100 g of washed resin (fines removed) in a 1,000-ml cylindrical separatory funnel which contained a small plug of glass wool to support the resin. The resin was converted to the formate form by slowly passing formic acid (88%) through the column until the eluent was chloride-free (determined by testing with silver nitrate). The column was then washed with 1 liter of demineralized water. Sixteen liters of fermented medium was passed slowly through the column. Ochratoxin A, which was found in the upper few millimeters of the column, was eluted with 1 to 2 liters of 10 M formic acid (in 50% aqueous methanol). Fractions (100-ml) of eluent were collected and assayed for ochratoxin A. Fractions containing ochratoxin A were combined.

The formate solution containing ochratoxin A was extracted four times with chloroform in a separatory funnel to give a combined chloroform extract of 1 liter or less. Thin-layer chromatography (TLC) procedures were used to determine the completeness of the extraction. The combined chloroform extract was concentrated to a volume of 50 to 75 ml under slightly reduced pressure at room temperature. This solution was then applied to a silica gel column (2 by 30 cm, 200 mesh, in chloroform), which was next washed with 100 ml of benzene. Ochratoxin was eluted from the column with approximately 200 ml of benzene-12% acetic acid solution. Progress of the ochratoxin band was monitored with a long-wave ultraviolet (UV) light (excessive exposure of ochratoxin to UV radiation should be avoided).

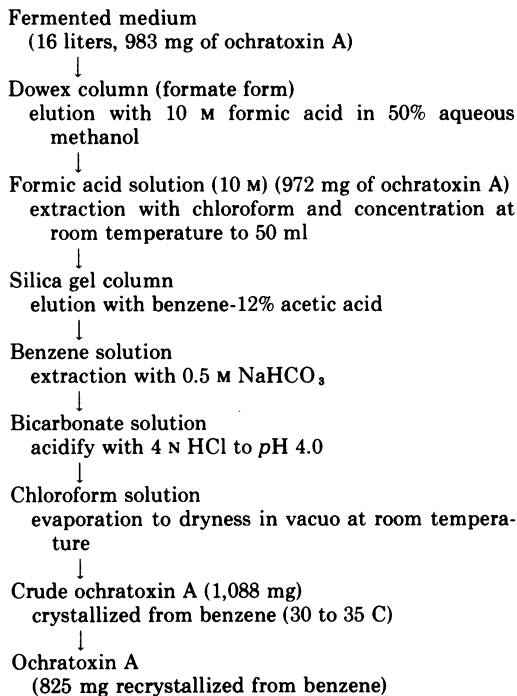


FIG. 1. General procedure for preparation of ochratoxin A from fermented media. The presence of ochratoxin A was determined by TLC procedures. The presence of crude ochratoxin A was determined by gravimetric procedures; it includes 1 mole of benzene per mole of ochratoxin A.

Presence of ochratoxin A in each 50-ml portion of eluent was determined by TLC. Fractions containing ochratoxin A were combined and extracted from the benzene solution in a separatory funnel by shaking with three successive 100-ml volumes of 0.5 M NaHCO₃. The bicarbonate solution was then acidified to pH 4 with 4 N HCl solution, and ochratoxin A was extracted with three 100-ml portions of chloroform in a separatory funnel. The completeness of extraction was determined by TLC. The chloroform solution of ochratoxin A was dried with anhydrous sodium sulfate and then evaporated to dryness under slightly reduced pressure at room temperature.

Crude ochratoxin was taken up in a slight excess of warm benzene (caution, the temperature must not exceed 38 C), and the solution was allowed to evaporate slowly at atmospheric pressure until crystallization of ochratoxin A occurred. Excess benzene was removed by filtration, and the ochratoxin crystals were washed with cold benzene. Remaining solvent was removed with a stream of dry nitrogen gas and finally by storing in a desiccator under

slightly reduced pressure. The final product contained 1 mole of benzene per mole of ochratoxin A.

To achieve efficiency in the procedure (Fig. 1), it was necessary to monitor the various extractions and elutions with TLC. Also, care was taken to avoid exposure of solutions of ochratoxin A to excessive light, UV radiation, and temperatures greater than 38 C.

Seven experiments gave yields of ochratoxin A ranging from 39 to 65 mg/liter of initial medium. In other experiments, higher yields were occasionally obtained. The mean for these seven experiments was 46 mg/liter of initial medium with a standard deviation of 10 mg/liter. In subsequent experiments, yields at the high end of the range were consistently obtained by monitoring ochratoxin production during the incubation period (time of maximum production of ochratoxin varied between the 7th and 8th day and declined thereafter). Fractions of the medium were periodically extracted with chloroform, and ochratoxin A was measured by TLC procedures, by optical densities at 333 nm, or by both. In this way, incubation could be terminated at the time of maximum yield. However, care must be taken with these measurements on extracts of crude media, since hydroxymellein may easily be confused with ochratoxin A; confirmation tests are usually necessary (1; J. H. Moore, Ph.D. thesis, Auburn Univ., Auburn, Alabama, 1971).

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LITERATURE CITED

1. Cole, R. J., J. H. Moore, N. D. Davis, J. W. Kirksey, and U. L. Diener. 1971. 4-Hydroxymellein: a new metabolite of *Aspergillus ochraceus*. *J. Agr. Food Chem.* 19:909-911.
2. Davis, N. D., J. W. Searcy, and U. L. Diener. 1969. Production of ochratoxin by *Aspergillus ochraceus* in a semisynthetic medium. *Appl. Microbiol.* 17:742-744.
3. Doupnik, B., and J. C. Peckham. 1970. Mycotoxicity of *Aspergillus ochraceus* to chicks. *Appl. Microbiol.* 19:594-597.
4. Ferreira, N. P. 1968. The effect of amino acids on the production of ochratoxin A in chemically defined media. *Antonie van Leeuwenhoek. J. Microbiol. Serol.* 34:433-440.
5. Lai, M., G. Semeniuk, and C. W. Hesseltine. 1970. Conditions for production of ochratoxin A by *Aspergillus* species in a synthetic medium. *Appl. Microbiol.* 19:542-544.
6. Lillehoj, E. B., A. Ciegler, and R. W. Detroy. 1970. Fungal toxins, p. 94-96. *In* F. R. Blood (ed.), *Essays in toxicology*. Academic Press Inc., New York.
7. Pitout, M. J. 1968. The effect of ochratoxin A on glycogen storage in the rat liver. *Toxicol. Appl. Pharmacol.* 13:299-306.
8. Pitout, M. J., and W. Nel. 1969. The inhibitory effect of ochratoxin A on bovine carboxy peptidase *in vitro*. *Biochem. Pharmacol.* 18:1837-1843.

9. Purchase, I. F. H., and J. J. Theron. 1968. The acute toxicity of ochratoxin A to rats. *Food Cosmet. Toxicol.* **6**:479-483.
10. Smalley, E. B. 1971. Effect of mycotoxins-ochratoxins and 12, 13 epoxytrichothecenes, p. 89-99. 32nd Minn. Nutr. Conf., Minneapolis, Sept. 20-21. University of Minnesota, St. Paul.
11. Yamazaki, M., Y. Maebayashi, and K. Miyaki. 1970. Production of ochratoxin A by *Aspergillus ochraceus* isolated in Japan from moldy rice. *Appl. Microbiol.* **20**:452-464.