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Ochratoxin A Production by Strains of *Aspergillus niger* var. *niger*

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In a survey of the occurrence of ochratoxin A (OA)-positive strains isolated from feedstuffs, two of the 19 isolates of *Aspergillus niger* var. *niger* that were studied produced OA in 2% yeast extract–15% sucrose broth and in corn cultures. This is the first report of production of OA by this species.

Ochratoxin A (OA) {(*R*)-*N*-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-L-phenylalanine} is a mycotoxin that is receiving increasing attention worldwide because of the hazard it poses to animal and human health. OA contaminates a variety of plant and animal products but is most often found in stored cereal grains. As a nephrotoxin, it has long been of particular importance to the poultry and swine industries. OA residues can enter the human food chain via their blood and meat products (1, 7, 11, 14, 15). Krogh (10) postulated some years ago that this mycotoxin was involved in Balkan endemic nephropathy; however, it seems that no clear causal relationship between them can be established (13). OA is now considered a potent carcinogen in mice and rats (11, 18).

OA was isolated originally as a metabolite from a strain of *Aspergillus ochraceus* in 1965. During subsequent years, six other species of *Aspergillus* section *Circumdati* (*A. ochraceus* group) have been identified as OA producers: *A. alliaceus*, *A. melleus*, *A. sulphureus*, *A. ostianus*, *A. petrakii*, and *A. sclerotiorum* (2, 21). The production of OA by *Eurotium herbariorum*, a member of *Aspergillus* section *Aspergillus* (*A. glaucus* group), has recently been reported (4). OA has become established as a metabolite of some *Penicillium* species as well (17).

In a screening of OA-producing strains belonging to *Aspergillus* and *Eurotium* species isolated from poultry mixed feeds and raw materials (corn, soya bean, and peas), 2 of 19 isolates of *A. niger* var. *niger* studied produced OA in liquid cultures as well as in corn cultures. The isolates were isolated from broiler mixed feed (strain A-75) and from soya bean (strain A-136) and were identified according to Klich and Pitt (9), who include in *A. niger* var. *niger* the species *A. tubingensis* and *A. ficcum*, according to the most accepted criteria of classification in *Aspergillus* section *Nigri*.

Dense conidial suspensions (0.1 ml) were inoculated onto 5 ml of YES broth (2% yeast extract, 15% sucrose) contained in 30-ml vials and then slanted to provide more surface area. After incubation for 7 days at 25°C, the vials were extracted with hot chloroform (20). Moistened yellow corn kernels (26 g of corn and 34 ml of water) were autoclaved in petri dishes at 121°C for 1 h on each of 2 consecutive days. Moistened corn kernels were also inoculated with 0.5 ml of conidial suspension. The cultures were incubated in the dark at 25°C for 6 weeks, dried at 50°C, ground, and extracted (12).

The production of OA was determined by thin-layer chromatography (TLC) and by high-pressure liquid chromatography (HPLC). Extracts were analyzed for OA by thin-layer chromatography using aluminum sheets (20 by 20 cm) of silica gel 60 without fluorescent indicator (no. 5553; Merck) and toluene–ethyl acetate–90% formic acid (6:3:1 [vol/vol/vol]) for development. OA was identified by comparison with appropri-

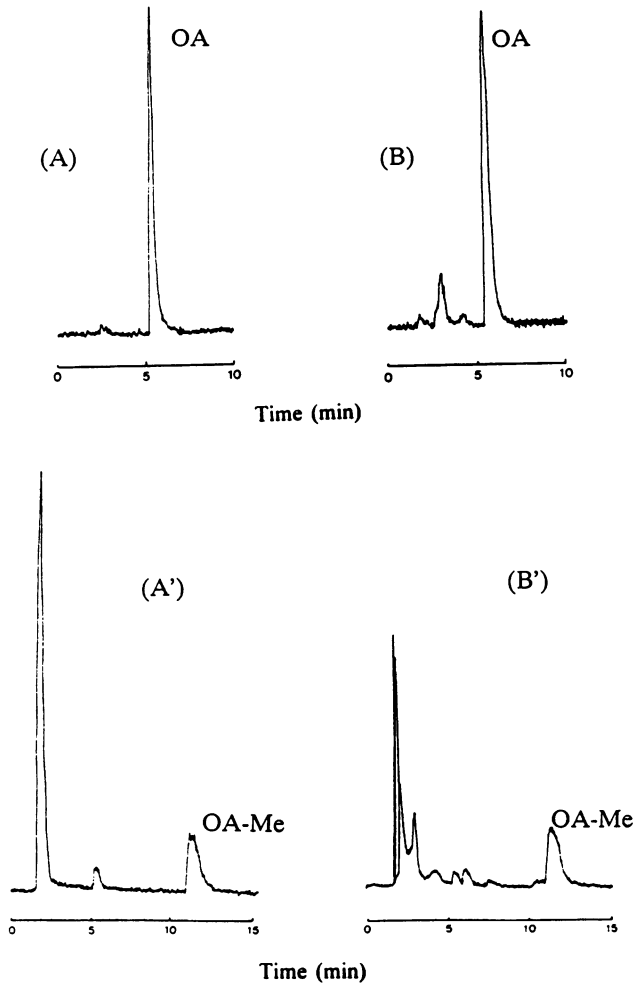

FIG. 1. HPLC chromatograms for OA standard (12.8 ng) (A), OA produced by *A. niger* var. *niger* A-136 on YES broth (17.82 ng) (B), and their corresponding methyl ester derivative OA-Me (A' and B'). Retention times: OA, 5.25 min; OA-Me, 11.30 min.

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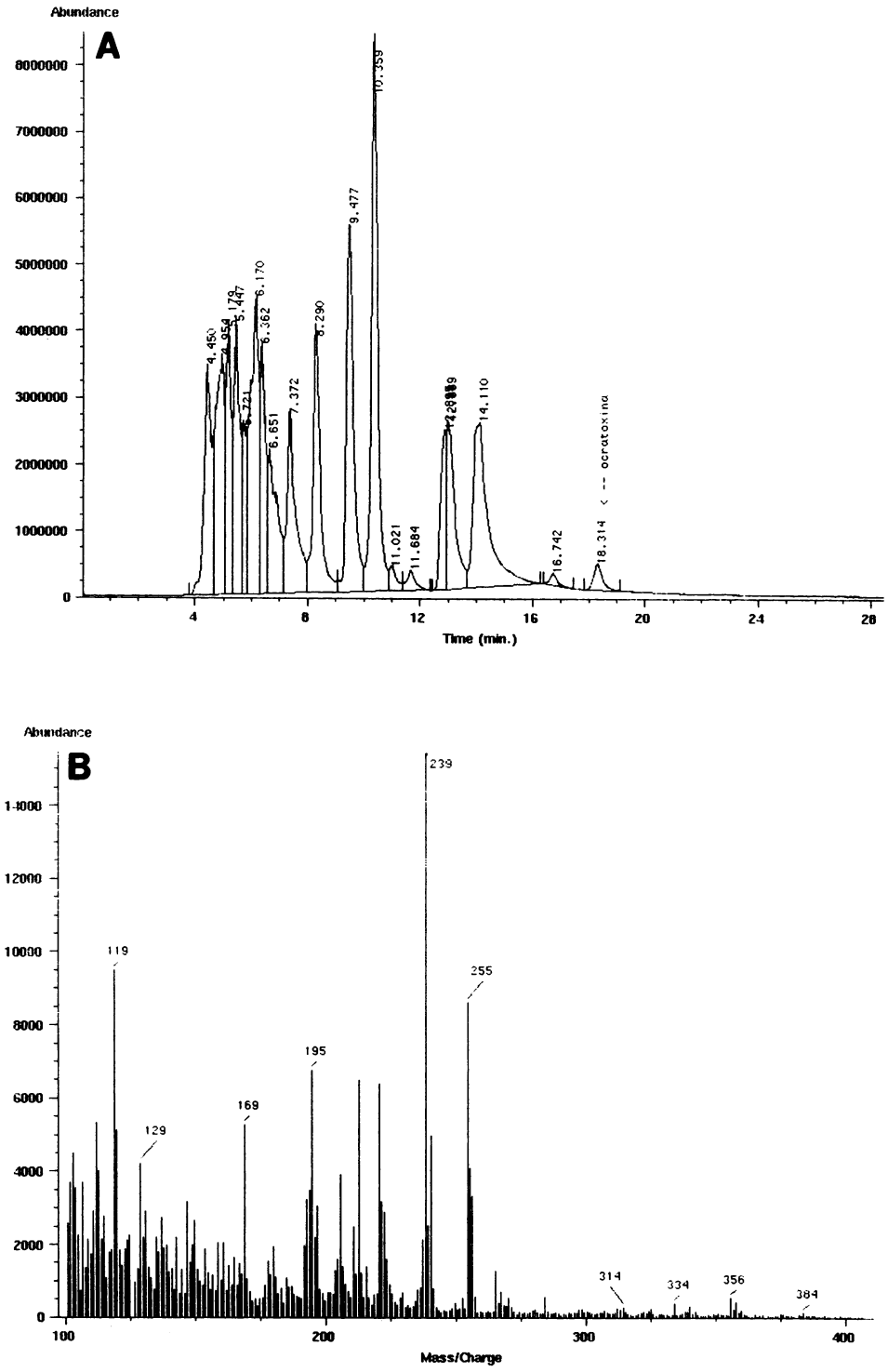

FIG. 2. (A) HPLC chromatogram for strain *A. niger* var. *niger* A-75 on YES broth; (B) mass spectrum of the peak eluting at 18.314 min shown in panel A. The mass spectrum was identical to those obtained with standard OA (these analyses were made at the Chemical Analyses Service of the Universidad Autónoma de Barcelona).

ate reference standards, including internal standards (Sigma Chemical Co., St. Louis, Mo.). A positive extract formed a green fluorescent spot under UV light with the same R_f as authentic OA. The fluorescence turned blue on treatment with ammonia, and spraying developed plates with $AlCl_3$ increased the fluorescence intensity (2).

The HPLC apparatus used was a model 500B-G (Konik, Barcelona, Spain) which had a loop of 20 μl and was equipped

with a spectrofluorescence detector (model 403; Konik) (excitation, 330 nm; emission, 460 nm) and a C_{18} column (Spherisorb S5 octyldecyl silane 2 [250 by 4.6 mm, 5-μm particle size], Phase Separations Ltd., Deeside, Clwyd, United Kingdom). The mobile phase was pumped at 1.0 ml/min and consisted of an isocratic program as follows: 57% acetonitrile, 41% water, and 2% acetic acid (1). HPLC analysis of positive extracts showed a peak at a retention time identical to that of standard OA. The identity of the OA peak was confirmed by spiking the extract with standard OA. OA production was again confirmed by preparing its methyl ester by treatment with 14% BF_3 in methanol (8) (Fig. 1).

Since this compound had not previously been reported as a metabolite of any species in *Aspergillus* section *Nigri*, mass spectrometric confirmation was obtained by HPLC-mass spectrometry particle beam interface (HP 1090, HP 5989X, HP 59980B). Both species grown on both media were confirmed by HPLC-mass spectrometry. The mass spectra were the same in a peak identical to that of standard OA (Fig. 2).

OA was quantified on the basis of HPLC fluorometric response compared with that of an OA standard. In YES cultures, *A. niger* A-75 and A-136 produced 0.23 and 0.59 μg/ml, respectively. In corn cultures, the OA concentrations found were 0.21 μg/g (strain A-75) and 0.36 μg/g (strain A-136). Although these strains can be considered weak producers under the conditions assayed, these concentrations are quite similar to those reported for some strains of *A. ochraceus* (6, 19) and *Eurotium herbariorum* (5). As far as we are aware, this is the first report of production of OA by *A. niger*.

A. niger consistently produces toxic naphthopyrones and malformins among other secondary metabolites; however, known toxic metabolites from *Aspergillus* section *Nigri* have not been found naturally occurring in cereals (4). The black aspergilli are worldwide in distribution, occurring on a great variety of substrates, including grains, forage products, spoiled fruits and vegetables, dairy products and other protein-rich substrata, and decaying vegetation in the field, and are widely used in industry for the production of organic acids, enzymes, and other products (3). *A. niger* on a variety of substrates has been investigated in detail and shows promise for production of proteins for human and animal consumption and for improving protein-poor diets (3, 16).

The results of this investigation indicate that the potential production of OA by this common fungus could be an unexpected hazard to human and animal health if such strains are used as starters, for example, in food industry. Enzyme preparations for food technology may present a similar hazard with regard to potential toxin residues.

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