

Production of Ochratoxins A and B on Country Cured Ham

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Two strains of *Aspergillus ochraceus* and six of *Penicillium viridicatum* isolated from country cured hams were screened for production of ochratoxins A and B. None of the isolated *P. viridicatum* strains yielded detectable amounts of ochratoxin A or B, whereas both strains of *A. ochraceus* produced ochratoxins A and B on rice, defatted peanut meal, and country cured ham. After 21 days of incubation on ham, one-third of the toxin was found in the mycelial mat on the ham surface, whereas two-thirds had penetrated into the meat to a distance of 0.5 cm.

During a recent survey on the mycoflora on country cured hams in the Southeastern United States, Sutic et al. (17) isolated two aflatoxin-producing strains of *Aspergillus flavus* Link ex Fries as well as other potential mycotoxin-producing aspergilli. Among these strains, one was identified as *A. ochraceus* Wilhelm. In a similar study, Strzelecki et al. (16) recovered two strains of *A. ochraceus* from country cured hams but were not able to show any production of ochratoxins.

Ochratoxin A and its dechlorinated analogue, ochratoxin B, are metabolites of several members of the *A. ochraceus* group (6) and of *Penicillium viridicatum* Westling (13), a mold which was also frequently found on country cured hams by Leistner and Ayres (8). The toxicity of ochratoxin A is well established, whereas ochratoxin B was first reported to be nontoxic (15). Later Peckham et al. (11) indicated that the toxicity of ochratoxin B was one-tenth that of ochratoxin A to day-old chicks. No carcinogenic effect has been observed for either toxin. Scott et al. (13) and Shotwell et al. (14) described cases of natural occurrence of ochratoxin A in moldy corn, wheat, and other agricultural products.

In the present investigation, country cured hams from curing plants in Georgia were surveyed for occurrence of *A. ochraceus*. Isolates of *A. ochraceus* and *P. viridicatum* from cured hams were then screened for production of ochratoxin A and B by using media known to be suitable for obtaining high-toxin yields.

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Since it was not known if cured ham itself could sustain ochratoxin formation, or how deeply the toxin or mold mycelium would penetrate if the surface of a whole ham were contaminated with *A. ochraceus* or *P. viridicatum*, these factors were also checked.

MATERIALS AND METHODS

Organisms. From five Georgia processing plants, 166 swabs were taken from 153 hams aged for 1 to 12 months. Sampling was restricted to colonies that appeared to have morphological and cultural characteristics of aspergilli. Each swab was inoculated initially on the Czapek-Dox agar plus 10% NaCl, thus favoring the growth of osmophilic species of *Aspergillus* which includes *A. ochraceus* (7), and then onto malt agar for final identification. From the 166 swabs, one strain of *A. ochraceus* (H 33) was found.

This strain, as well as *A. ochraceus* D-1, isolated by Sutic et al. (17) from cured ham, and *A. ochraceus* NRRL 3174, a known ochratoxin producer (3), were incubated on slants of Czapek-Dox agar for 10 days at 27 C and then stored at 4 C. Six strains of *P. viridicatum*, M 110, M 133, M 165, M 206, M 218, and M 240, all recovered from cured hams by Leistner and Ayres (8), were received as soil cultures and transferred on Czapek-Dox agar as above. *P. viridicatum* (ATCC 18411) which, according to Van Walbeek et al. (19), produces ochratoxin A, was included as reference.

Culturing. (i) On rice, defatted peanut meal, and corn. Spores (3×10^8) of each strain of *A. ochraceus* and *P. viridicatum* and 75 ml of sterile water were added to 150 g of rice or defatted peanut meal in 500-ml Erlenmeyer flasks and incubated at 25 to 27 C for 14 days. In addition, *P. viridicatum* was cultured on popcorn under the same conditions. These conditions were reported by Schindler and Nesheim (12) to yield maximal amounts of toxin. After steaming the

cultures to facilitate extraction of ochratoxins, they were transferred to Mason jars and extracted.

(ii) **On country cured ham to screen for ochratoxin production.** Boneless slices of fully aged country cured ham (0.5–1.0 cm thick) were procured from various commercial curing plants. Excessive fatty parts were removed and the slices were cut to a weight of 100 to 150 g. They were surface sterilized by dipping them into 1% NaOCl solution for 1 min, rinsing with sterile water, and blotting dry with sterile cheese cloth.

For inoculation, the slices were swabbed with 0.5 to 1.0 ml of a spore suspension containing 10^6 spores per ml, thus obtaining a spore load of 0.5×10^6 to 10^6 spores per slice. The ham slices were then suspended by a string in sterile 1-qt (0.946-liter) Mason jars which were covered with three layers of no. 1 Whatman filter paper instead of Mason lids. The jars were incubated for various lengths of time at 5, 15, 25, 30, and 37 C and at a relative humidity of 70 to 75%. If necessary, saturated aqueous NaCl solution (relative humidity 75% at 20 C [10]) was added to the jars. After incubation, the ham slices were cut into small pieces for extraction.

(iii) **On country cured ham to determine toxin and mold penetration.** From the center section of fully cured and aged hams, slices (5 cm thick) each weighing about 1,000-g were cut, surface sterilized as described above, and placed into sterile culturing chambers. The top surface (crossing the bone, 150 to 180 cm²) was inoculated with approximately 10^3 spores per cm², whereas the edges were kept sterile by repeatedly cleaning them with NaOCl solution. Cultivation was at 25 C and 70 to 75% relative humidity for 21 days. The mycelial mat was then scraped from the surface and the slices were cut into layers (0.5 cm thick) which were assayed individually for ochratoxins. Also, slabs were cut from the slices in different depth, again surface sterilized, and incubated on rose bengal-streptomycin agar RBM-2 (18) at 25 C.

Assays. Official methods of the Association of Official Analytical Chemists (1) were used to determine moisture and salt content of cured hams.

To quantitate ochratoxins, all cultures were extracted with chloroform by using a Sorvall high-speed blender. The crude extracts were filtered through diatomaceous earth and concentrated to 50 ml. A clean-up step with column chromatography by the procedures proposed by Eppley (4) followed. The ochratoxins in the purified extracts were separated by thin-layer chromatography on Adsorbosil-1 (Applied Science Laboratories, State College, Pa.) with toluene-ethyl acetate-formic acid 5:4:1 (vol/vol/vol) as developing solvent (4). To exclude possible interference of 4-hydroxymellein with ochratoxin A, chloroform-acetone 93:7 (vol/vol) was used as a second solvent system (9). A Photovolt fluorodensitometer was used to compare intensity of fluorescence of the samples with that of standards obtained from the Bureau of Food Sanitation, Food and Drug Administration, Washington, D.C. It was possible to improve the sensitivity of this method by exposing the thin-layer chromatography plates to ammonia fumes for 2 to 4 min, a treatment which changes the fluorescence

of both ochratoxin A and B from blue-green to intense blue. The reaction with ammonia is specific for both ochratoxin A and B. Additional confirmation was obtained by extracting the purified extract with 0.1 M aqueous sodium bicarbonate solution, acidifying with 2 M hydrochloric acid, and re-extracting with chloroform (4). This extract was concentrated and chromatographed as before.

RESULTS AND DISCUSSION

As the results of the screening tests in Table 1 indicate, both isolates of *A. ochraceus* recovered from country cured ham were able to produce ochratoxins A and B. Toxin yields were lower than those observed on shredded wheat by Schindler and Nesheim (12). Whereas *P. viridicatum* (ATCC 18411) produced ochratoxin A as expected, none of the isolates of *P. viridicatum* from cured hams formed detectable amounts of either ochratoxin A or B. This contrasts somewhat with the results of Scott et al. (13), who found only 5 nonproducers among 27 strains of *P. viridicatum* isolated from various grains, mixed feed, beans, and peanuts.

Since the isolates of *P. viridicatum* from cured ham did not yield any toxin on rice, peanut meal, or corn, only *A. ochraceus* was grown on aged hams. Table 2 shows that again all three strains of *A. ochraceus* produced ochratoxins A and B. The temperature optimum for toxin production by strain H 33 was 25 to 30 C; very little toxin was recovered at 15 C and none was recovered at 5 or 37 C, at which temperatures only poor mycelial growth and almost no sporulation occurred. At 25 C, strain H 33 produced more toxin on hams containing 45% moisture than on those having 55%, the latter being above the equilibrium moisture at 75% relative humidity. These hams therefore lost 5% moisture during incubation. *Aspergilli* tend to show optimal growth at comparatively low water activities (8). Culturing at 90 and 100% relative humidity in ambient air resulted in poor growth and successful competition from other molds and bacteria present in hams. In aging rooms of commercial plants, 75% relative humidity prevails.

In the study on mold and toxin penetration into ham (55% moisture, 5.1% NaCl), mycelial growth was observed as deep as 1 cm along the binding tissue where the meat often split open during the incubation period (21 days at 25 C and 75% relative humidity). In the lean muscle, growth occurred to a depth of approximately 0.5 cm. This figure was not very consistent, since contamination from other locations on the hams was difficult to prevent when the ham slabs were prepared for incubation. One-third (7 μ g)

TABLE 1. Production of ochratoxins A and B by *A. ochraceus* and *P. viridicatum* incubated on rice and defatted peanut meal

Mold	Rice ^a		Defatted peanut meal ^a	
	Ochratoxin A ($\mu\text{g}/\text{kg}$)	Ochratoxin B ($\mu\text{g}/\text{kg}$)	Ochratoxin A ($\mu\text{g}/\text{kg}$)	Ochratoxin B ($\mu\text{g}/\text{kg}$)
<i>A. ochraceus</i>				
D-1	6,000 ^b	15,000	1,000	1,400
H-33	151,200	22,300		
NRRL 3174	5,800	1,500	5,500	3,700
<i>P. viridicatum</i>				
M 110, M 133, M 165, M 206, M 128, and M 240	ND ^c	ND	ND	ND
ATTC 18411	80	Traces		

^a One hundred fifty grams with 75 ml of water; incubated at 25 C for 14 days.

^b Mean of two experiments.

^c ND, Not detectable (less than 1 μg [15 $\mu\text{g}/\text{kg}$]).

TABLE 2. Production of ochratoxins A and B by *A. ochraceus* on country cured ham

Strain	Initial ham composition ^a		Incubation ^b		Ochratoxin A ($\mu\text{g}/\text{kg}$)	Ochratoxin B ($\mu\text{g}/\text{kg}$)
	Moisture (%)	NaCl (%)	Days	Temp (C)		
NRRL 3174	45	4.1	10	25	7,900 ^c	4,200
D-1	45	4.1	10	25	6,600	4,500
H-33	45	4.1	10	25	5,000	3,500
H-33	55	5.1	20	5	ND ^d	ND
H-33	55	5.1	20	15	Traces	ND
H-33	55	5.1	10	25	770	120
H-33	55	5.1	10	30	390	80
H-33	55	5.1	10	37	ND	ND

^a One hundred- to one hundred fifty-gram pieces.

^b Relative humidity, 70 to 75%.

^c Mean of two experiments.

^d ND, Not detectable (less than 2.5 μg [25 $\mu\text{g}/\text{kg}$]).

of the total ochratoxin A produced was found in the mycelial mat on the surface of the ham, two-thirds (14 μg) was found in the upper 0.5-cm meat layer (260 μg of ochratoxin A per kg of meat), only traces were found in the second 0.5 cm, and none was found in the deeper layers. Ochratoxin was detected only in the layer in which mycelial growth was also observed. Ochratoxin apparently penetrates into the meat along with the mycelial growth, whereas physical diffusion of the toxin is limited, at least during the 3-week period of this experiment. This is different from cultures of *A. ochraceus* in liquid media, where a large proportion of toxin is always found in the culture filtrate. In studies of aflatoxin in bread, Frank (5) recovered the

toxin only in zones where mycelial growth occurred.

There is evidence that *A. ochraceus* is able to produce more than negligible amounts of ochratoxins A and B on country cured hams under conditions which are often encountered in commercial curing plants. Mycelial growth is not restricted to the ham surface, and toxin can penetrate as far as 0.5 cm into the muscle of the meat. The three strains of *A. ochraceus* used in this study also produced penicillic acid on cereals. Penicillic acid does not seem to be a problem on meat because it reacts easily with amino acids to form much less toxic or nontoxic compounds (2). None of the strains of *P. viridicatum* isolated from hams produced measurable amounts of ochratoxin A or B. However, besides also producing penicillic acid (2), *P. viridicatum* is a source of the mycotoxin citrinin (13). Its occurrence on aged hams and its importance are still to be determined.

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