Tremorgenic Mycotoxins Produced by *Aspergillus fumigatus* and *Penicillium crustosum* Isolated from Molded Corn Implicated in a Natural Intoxication of Cattle

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Aspergillus fumigatus and *Penicillium crustosum* were isolated from a sample of corn that was implicated in a natural intoxication involving a herd of beef cattle. When cultured on corn, both isolates were found to be toxigenic. The *A. fumigatus* isolate produced verruculogen as well as several toxic clavine alkaloids and the *P. crustosum* isolate produced penitrem A and related tremorgens. A small sample of the corn was analyzed for the presence of penitrem A, verruculogen, and related tremorgens, but these were not detected.

A suspected intoxication involving a herd of beef cattle on a Michigan farm during the spring of 1980 prompted us to investigate the toxin-producing potential of two fungal species isolated from corn being used as feed. Approximately 120 animals, consisting of two groups averaging 250 and 500 kg/animal, respectively, were affected and exhibited abnormal behavior, incoordination, and ataxia, and there were 11 mortalities. The cattle were feeding on hay silage with a top dressing of high-moisture corn. Onset of clinical signs was rapid and evident 10–14 days after being on the suspect feed. A sample of the corn was submitted for a mold count (Woodson—Tenent Laboratories, Inc., Dayton, OH) and found to contain 20,000 propagules/g of a *Penicillium* species and 2000 propagules/g of *Aspergillus fumigatus* with *Aspergillus flavus* absent. The corn sample we received appeared to be of relatively good quality and the fungal propagules present in the sample were apparently superficially associated with the sample. Presumably, these propagules were carried as dust from a fermentation site elsewhere in the lot of corn.

A. fumigatus is known to produce toxic metabolites, including tremorgens of the fumitremorgin–verruculogen group (Cole, 1981). The fungus has been implicated in naturally occurring toxic syndromes of beef and dairy cattle in which animals suffered from irritability, incoordination, behavior change, and death (Cole et al., 1977; Smith and Lynch, 1973). Penitrem A, a tremorgenic toxin produced by species of *Penicillium*, has been shown to be the causative agent of a natural intoxication of dogs that had consumed moldy cream cheese (Arp and Richard, 1979; Richard and Arp, 1979), and it was implicated in a toxic syndrome of sheep and horses (Wilson et al., 1968). Therefore, *A. fumigatus* and *Penicillium* species, as contaminants of feed, have the potential to produce disease syndromes in which affected animals exhibit clinical signs that include tremors, irritability, incoordination, dramatic change in behavior, and death.

We now report the toxin-producing potential of isolates of *A. fumigatus* and *P. crustosum* isolated from corn associated with the disease syndrome of cattle described above.

MATERIALS AND METHODS

Analysis of Suspect Corn. A 2000-g sample of corn being used as feed for the affected animals was analyzed for the presence of tremorgens by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The entire sample of corn was ground with ethyl acetate in a Waring Blendor for 4 min and filtered, and the extract was concentrated to an oily residue with a rotary evaporator at 60 °C. The residue was then brought to 300 mL with hexane and partitioned 2 times against an equal volume of 80% aqueous methanol. The aqueous phases were combined and methanol was removed under vacuum, leaving an aqueous residue. This was extracted with an equal volume of dichloromethane, which was filtered through anhydrous sodium sulfate, concentrated to dryness, and redissolved in 2 mL of methanol. This preparation was used for TLC analysis by applying 1–5 μL (in 1-μL increments) to precoated silica gel 60 F-254 plates (5 by 10 cm; EM Laboratories, Inc., Elmsford, NY), which were developed in solvent systems of toluene–ethyl acetate–formic acid, 5:4:1 v/v/v, and chloroform–acetone, 93:7 v/v. Plates were spotted with internal and external standards of penitrem A, verruculogen, fumitremorgin A, fumitremorgin B, and fumitremorgin C. The concentration of each standard was 50 μg/mL, and the minium detectable limit of each was 50 ng. This would correspond to a minimum detectable contamination level of 10 ppb in the suspect corn. Developed plates were sprayed with 50% ethanolic sulfuric acid, heated, and viewed under long wave ultraviolet light to detect the verruculogen–fumitremorgin compounds or sprayed with 1% *p*-(dimethylamino)benzaldehyde in ethanol followed by ethanolic sulfuric acid to detect penitrems.

For HPLC analysis a method was developed based on the procedure for the penitrems reported by Maes et al. (1982). Further sample preparation was carried out by reducing the 2-mL methanol preparation used for TLC analysis to dryness and redissolving it in 4 mL of benzene. This was applied to a Sep-PAK silica cartridge (Waters Associates, Milford, MA) and eluted with 10 mL of benzene followed by 10 mL of methanol. The methanol eluate was reduced in volume to 2 mL and subjected to reverse-phase chromatography using a Waters Associates HPLC system including an M6000A pump, a Model 720 data module, a Model 450 variable-wavelength UV detector at 296 nm, and a Z-module with a 5 mm × 10 cm Radial-PAK C_{18} cartridge. The mobile phase consisted of methanol–water, 72:28, and tremorgen standards were separated and detected in 10 min with a 2 mL/min flow rate.

Isolation and Culture of Fungi. Fungi were isolated from the suspect corn on malt agar plates incubated at 25 and 37 °C. Cultures were maintained on potato dextrose agar (PDA) plates at 5 °C after 4–7 days of growth.

The two primary isolates obtained were cultured on a medium of cracked corn to evaluate their toxin-producing potential. *A. fumigatus* and *Penicillium crustosum* were

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grown in 10 Fernbach flasks (2.8 L), each containing 200 g of corn and 70 mL of water, for 2 weeks with incubation temperatures of 37 and 25 °C, respectively.

Extraction, Detection, and Purification of Toxins. The *A. fumigatus* cultures were extracted by homogenization with an equal volume of chloroform, filtered through cheesecloth, and then filtered through anhydrous sodium sulfate. The extract was reduced in volume on a rotary evaporator to 500 mL and partitioned 3 times against equal volumes of 0.5 M HCl solution. The aqueous phases were combined, adjusted to a pH of 10.0 with sodium carbonate, and partitioned 3 times with 200 mL of chloroform. The chloroform extracts of the basic fraction were combined, washed with distilled water, filtered through anhydrous sodium sulfate, and concentrated under vacuum. The neutral chloroform extract was also filtered through sodium sulfate and concentrated. The basic fraction was subjected to TLC analysis in two different systems for preliminary identification of alkaloids based on comparison with various standards. Normal-phase precoated silica gel 60 F-254 plates were developed in chloroform–methanol, 9:1 v/v, and reverse-phase precoated KC_{18} plates (Whatman, Inc., Clifton, NJ) were developed in methanol–water, 85:15 v/v. Plates were sprayed first with 1% (*p*-dimethylamino)-benzaldehyde in ethanol and then with 50% ethanolic sulfuric acid followed by heating for 2 min at 100 °C to detect the alkaloids.

The neutral fraction was subjected to column chromatography to purify what TLC analysis showed to be verruculogen or a verruculogen-related metabolite. Columns were monitored by TLC for pooling of the pertinent fractions. The neutral extract was dissolved in benzene and applied to a silica gel column (9.5 × 17 cm) that was eluted sequentially with 2 L each of benzene, ethyl ether, ethyl acetate, and acetone. The ether fraction was reduced in volume and chromatographed on a 3.5 × 40 cm silica gel column with a gradient of benzene to ether with the collection of 180 17-mL fractions. Fractions 101–131 were combined, concentrated, and applied to a Florisil column (1.5 × 30 cm) eluted with 500 mL of 5% ethyl acetate in benzene. Fractions 19–27 were combined, concentrated to near dryness, and dissolved in 10 mL of methanol for crystallization of the purified metabolite.

The *P. crustosum* cultures were homogenized twice with equal volumes of ethyl acetate, filtered through cheesecloth and anhydrous sodium sulfate, and concentrated under vacuum. Because TLC analysis indicated the probable presence of penitrem A in the extract, column chromatography was again utilized to purify the metabolite. The oily residue was applied to a silica gel column (4.5 × 50 cm) packed in benzene and eluted with a linear gradient of benzene to ethyl ether. One hundred seventy-six 17-mL fractions were collected, and TLC analysis showed that fractions 35–58 contained the metabolite of interest. These fractions were combined, concentrated to near dryness, and dissolved in minimal tetrahydrofuran. This was applied to a C_{18} reverse-phase column (1.5 × 10 cm) that was eluted with a linear gradient of 50% aqueous methanol to methanol. As a result of TLC analysis, fractions 75–87 were combined, concentrated to dryness, and dissolved in 10 mL of methanol for crystallization.

Identification of purified toxins was based on chromatographic characteristics (TLC and HPLC) as well as ultraviolet (UV), infrared (IR), and low-resolution mass spectral analyses.

RESULTS AND DISCUSSION

Both TLC and HPLC analyses of the suspect corn for the presence of penitrem A, fumitremorgins A, B, and C, and verruculogen were negative.

Only two species of fungi were isolated in large numbers from the suspect corn. They were identified as *A. fumigatus* Fres. and *P. crustosum* Thom. Samson et al. (1976) and Ramirez (1982) considered *P. crustosum* to be synonymous with *P. verrucosum* var. *cyclopium*. Pitt (1979), however, expressed the opinion that *P. crustosum* was a distinctive species. This isolate caused a distinct brown rot when inoculated into sound Golden Delicious apples (2.4-cm diameter in 8 days at 25 °C) and oranges (Florida navel, 2.5-cm diameter in 8 days at 25 °C), and we consider it to be typical of *P. crustosum* Thom.

The *A. fumigatus* isolate produced several toxic metabolites when grown on corn. When the basic partition was analyzed by TLC with standards of several alkaloid mycotoxins, the following compounds were determined to be present in the extract in order of decreasing quantities: fumigaclavine C, fumigaclavine A, and festuclavine. These calvine alkaloids have been reported on other occasions from *A. fumigatus* (Spilsbury and Wilkinson, 1961; Yamano et al., 1962; Cole et al., 1977), and Cole et al. (1977) showed that fumigaclavine C was toxic to day-old cockerels with an LD_{50} of 150 mg/kg when dosed orally. The toxicity of fumigaclavine A and festuclavine is not known.

The metabolite purified from the neutral fraction of the *A. fumigatus* extract and crystallized from methanol was identified as verruculogen (Fayos et al., 1974) by comparing its UV, IR, mass spectra, TLC, and HPLC characteristics with those of authentic verruculogen. Verruculogen is a tremorgenic mycotoxin known to be produced by several species of *Penicillium* as well as *Aspergillus caespitosus* and *A. fumigatus* (Cole, 1981). Cole et al. (1977) reported a naturally occurring disease syndrome of beef cattle being fed silage contaminated with *A. fumigatus*, and under controlled experimental conditions, they fed cattle crude extracts of the *A. fumigatus* isolate, which produced the same clinical signs, including irritability and muscle tremors, that were observed in the field case. That extract also contained verruculogen as well as some of the clavine alkaloids reported here. It was concluded that the tremorgenic metabolites of *A. fumigatus* were at least involved in the neurological signs of that syndrome and may have been responsible for the other clinical signs.

Extracts of the *P. crustosum* isolate yielded fine crystals of penitrem A. This identification was confirmed by chromatographic, UV, IR, and mass spectral comparison of the crystals with authentic penitrem A.

Although tremorgens were not detected in the sample of corn analyzed, they cannot be ruled out as causing or contributing to the observed intoxication. In such cases where relatively small samples of suspect feed are available for analysis, often the most heavily contaminated portion of the feed has been consumed or is missed in the sampling process. The presence of relatively large numbers of fungal propagules in the corn sample that appeared to be of good quality strongly suggests that the actual fermentation occurred in a different part or different parts of the lot of corn. The propagules could therefore have been carried to other parts of the lot of corn as dust. *Penicillium* spp. and *A. fumigatus* have often been shown to be prolific producers of tremorgenic toxins, and their presence in the suspect corn and ability to produce such toxins when cultured on corn illustrate again the potential economic significance of mycotoxins generally and tremorgens specifically.

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Registry No. Verruculogen, 12771-72-1; penitrem A, 12627-35-9; fumigaclavine C, 62867-47-4; fumigaclavine A, 6879-59-0; festuclavine, 569-26-6.

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Nutrient Composition of Chinese Vegetables

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Data on the levels of water, protein fat, sugars (glucose, fructose, sucrose), starch, dietary fiber, organic acids, ash, sodium, potassium, calcium, iron, magnesium, zinc, vitamin C, thiamin, riboflavin, niacin, carotenes, and energy content are reported for 15 Chinese vegetables—Chinese chives, Chinese spinach, Chinese white cabbage, mustard cabbage, Chinese flowering cabbage, Chinese cabbage, garland chrysanthemum, water spinach, watercress, hairy melon, wax gourd, angled luffa, bitter melon, bean sprouts, and yard-long beans.

Vegetables traditionally consumed by the Chinese population in many countries are now consumed more frequently by people of non-Chinese origin, not only in Asia but also in Western countries along with the increased general popularity of Chinese food. However, there are few data in the literature on the nutrient composition of these Chinese vegetables. Vitamin C, crude fiber, sugar, and dry matter of the major Chinese cabbage variety (*Brassica pekinensis*) have been reported [e.g., AVRDC (1975, 1980)], but in other studies [e.g., Wuensch (1975) and Kayukova, 1977) there is inadequate identification of what botanical species is meant by "Chinese cabbage". Germinated mung bean sprouts [*Vigna radiata* (L.) R. Wicz (*Phaseolus aureus roxb.*; *phaseolus radiatus* L.; *Phaseolus sublobatus* Roxb.)] have been examined for protein content (AVRDC, 1975, 1976, 1979), with considerably more compositional data available on the unsprouted bean seed [e.g., Del Rosario et al., 1980; Creswell, 1981). Data available on mustard cabbage (*Brassica juncea*) is mostly on the composition of the seeds (Gambhir et al., 1979; Singh et al., 1979) rather than the leafy vegetable (Sreeramula, 1982). There are a number of studies on various vegetables that only report values for a single nutrient, particularly protein (Grubben, 1975; Rodriguez et al., 1975; Bruemmer, 1980), and Chen et al. (1982) have determined neutral detergent fiber in a number of vegetables. The most comprehensive data on Chinese vegetables are contained in the food tables produced for use in East Asia (FAO, 1972), but as with many national or regional food tables, the values are an amalgum of data from various unknown sources without any guide as to the reliability of individual values. In this study we have analyzed 15 types of Chinese vegetables grown in market gardens in Sydney, Australia, for proximate composition and a range of vitamins and minerals.

MATERIALS AND METHODS

Two samples each of 15 types of vegetable were obtained from a market garden in Sydney, Australia, as commercially mature produce either in March (summer grown) or July (winter grown) during 1982. On arrival at the laboratory, each food was identified, initially with reference to Dahlen and Phillipps (1980) and confirmed with the procedure of Nicholas (1974). The inedible portion was removed and the proportion of edible weight determined. The edible portion was homogenized in a blender. Samples were removed for determination of total vitamin C by the

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