Production and Biological Activity of Secalonic Acid D

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Twenty isolates of *Penicillium oxalicum* produced secalonic acid as their major secondary metabolite. Fermentation conditions were determined for toxin production in grain and liquid media. The 50% lethal dose value for mice ranged from 26.5 to 51.7 mg/kg dependent on animal strain and sex, males being more susceptible than females. Secalonic acid was nontoxic and nonteratogenic to the chicken embryo and exhibited poor antibiotic properties. Its potential role in mycotoxicoses is discussed.

Extracts of *Penicillium oxalicum*, a common fungus in cereals, has been reported to be toxic to laboratory animals (2, 8, 9). Steyn (9) identified secalonic acid (SA) as the major toxin produced by five strains of this species. At the Northern Regional Research Center, D. Fennell isolated *P. oxalicum* and *Aspergillus flavus* as the principal fungi contaminating corn in Iowa (5). We isolated *P. oxalicum* and *Fusarium moniliforme* from corn which was associated with the deaths of horses and pigs in Louisiana; animals which died exhibited pulmonary edema and excessive fluid in the body cavities. The horses had foamy blood coming from the nostrils.

We examined 20 isolates of *P. oxalicum* for their ability to produce SA, determined optimal conditions for toxin production, and evaluated acute toxicity of this compound to mice and chicken embryos.

MATERIALS AND METHODS

Cultures. Cultures were obtained either from the Northern Regional Research Center culture collection or from suspect corn after the kernels were sterilized with 2% sodium hypochlorite and plated onto potato dextrose agar. Isolates were maintained on Blakeslee malt extract agar slants.

For fermentation studies, 300 g of grain plus 100 ml of water was sterilized in 2.8-liter Fernbach flasks for 20 min and inoculated, two flasks per agar slant. Incubation was under the various conditions noted below.

Analyses. On completion of fermentation, the molded grains were steeped in methylene chloride for 1 to 3 days and filtered, and the residual solids were again extracted with the same solvent in a large Waring blender. Extracts were combined, dried with sodium sulfate, and concentrated by flash evaporation to a known volume. SA concentration was determined by thin-layer chromatography on Brinkmann pre-coated plates (Silica Gel 60, F-254, 0.25 mm thick) by

using benzene-ethyl acetate-formic acid (100:40:10) for developer. SA standards of 0.5 to 5 μ g were spotted, and the degree of quenching under shortwave ultraviolet light was compared to that given by test samples. Each plate was read twice by two individuals, and the four readings were averaged.

Toxin purification. For production of purified toxin, the above procedures were followed with cracked corn as the substrate. After 12 to 14 days of static incubation at 28°C, the fermented corn was extracted twice with methylene chloride, and the extractant was dried with sodium sulfate and concentrated by flash evaporation to a small volume. The oily concentrate was added to cold hexane and permitted to stand overnight in the cold, and the yellow precipitate was filtered off. The yellow solids were refluxed in the minimum volume of chloroform required for solution from which pale yellow SA needles crystallized out upon slow cooling. Two additional recrystallizations were followed with melting point, infrared, ultraviolet, nuclear magnetic resonance, and mass spectral analyses for purity and compound identification.

Biological tests. Adult mice between 30 and 35 g were used in all trials. Toxin was dissolved in dimethyl sulfoxide, and no more than 0.1 ml of solvent was injected. Lung, heart, liver, kidneys, and other grossly abnormal organs from injected mice were fixed in Lavdousky solution (formaldehyde, ethanol, acetic acid) for histopathology. Fifty percent lethal dose values and their 95% confidence limits were determined by the Weil method (10).

For chicken embryo assays we followed previously described procedures (3), initially dissolving the toxin in acetone, adding the solution to cottonseed oil, and removing the acetone by flash evaporation. Solutions also were made in 0.1 M NaHCO₃.

For antibiotic assays we utilized the procedures of Lindenfelser and Ciegler (6).

RESULTS

Production. Average yields of crystalline SA of 2.6, 1.9, and 2.1 g of toxin per kg of corn were attained in three fermentations of 9 kg of corn

each of *P. oxalicum* NRRL 5209. Ultraviolet, infrared, and nuclear magnetic resonance spectra and melting point (254 to 255°C; from CHCl₃) values corresponded to the data reported by Steyn (9) and high-resolution mass spectra matched the analyses of Howard and Johnstone (4) (*m/e* 638; C₃₂H₃₀O₁₄). Nuclear magnetic resonance analyses of crystalline preparations obtained from fermentations by *P. oxalicum* NRRL 5209, LA-2, and NRRL A 18620 corresponded to literature values for SA D (4, 9).

All 20 screened *P. oxalicum* strains that had been isolated from a variety of sources and geographical areas synthesized SA (Table 1). However, nuclear magnetic resonance was only established for the three isolates as noted above. Production ranged from 40 mg to over 2 g/kg of corn after 12 days of fermentation. Maximum yields were given by NRRL 5209 between 2 to 3 weeks of fermentation at 28°C; lower yields resulted at 20 and 37°C (Table 2). Similarly, still culture was advantageous over shake culture (Table 3), and cracked corn was superior to other grains and oilseeds (Table 4) for toxin production.

Excellent SA yields were realized in YES medium (2% yeast extract, 10% sucrose) (2.8 g of SA per liter), although there was considerable yield decrease when the sucrose concentration was lowered to 4% (40 mg of toxin per liter). Still culture proved superior to shake culture, and

TABLE 1. SA D production by various strains of *P. oxalicum*

NRRL isolate	Culture source	SA D (mg/kg of corn)
A 18968	Wheat	412
A 22691	Corn (Illinois)	412
A 18620	Wheat	2,060
A 20426	Corn	824
A 22679	Corn (Iowa) ^a	1,648
A 18958	Wheat	824
A 18886	Wheat	824
A 20153	Corn (Missouri)	84
A 22658	Corn (Iowa) ^a	40
La-1	Corn grown in Texas, shipped to Louisiana ^b	1,152
La-2		
La-3		
La-4		
La-5		
5209	Corn (South Africa)	1,890
A 23244	Prairie grass	206
A 23216	Prairie grass	356
A 23217	Prairie grass	206
A 23245	Prairie grass	154
A 23215	Prairie grass	154

^a Corn also contaminated with *A. flavus* and aflatoxin.

^b Corn probably involved in mycotoxicoses in pigs and horses.

TABLE 2. SA D production on corn at three temperatures by *P. oxalicum* NRRL 5209

Day	g of SA D/kg of corn at:		
	20°C	28°C	37°C
7	0.1	0.6	<0.1
10	0.5	1.5	0.1
12	— ^a	2.4	0.1
14	1.1	2.6	0.2
21	1.7	3.4	—
28	2.8	—	—

^a —, Nondetectable.

TABLE 3. SA D production on corn by *P. oxalicum* NRRL 5209: still versus shake culture

Day	Still (g/kg)	Shake ^a (g/kg)
5	0.3	<0.1
7	0.9	0.3
11	1.5	1.0
13	2.0	1.1
15	2.8	0.8
21	2.4	1.2

^a Flasks were shaken at 250 rpm.

TABLE 4. SA D production by *P. oxalicum* NRRL 5209 on various agricultural commodities^a

Commodity	mg of toxin per kg
Barley	818
Minute rice	206
Sorghum	989
Long grain rice	783
Brown rice	1,075
Wheat	1,071
Soybean	103
Corn	2,318
Corn grits	412

^a Two weeks of still incubation at 28°C. Data are the averages of duplicate samples with the greatest variation being $\pm 20\%$.

28°C was advantageous over 37°C (Table 5). SA was detected only in the mycelium with no detectable amount in the supernatant.

Biological tests. The 50% lethal dose values (intraperitoneal) at the 95% confidence limit were as follows: Charles River CD1 mice, 36.7 mg/kg (range, 30.5 to 44.2 mg/kg) for males and 51.7 mg/kg (range, 45.2 to 59.1 mg/kg) for females; Texas (ICR) male mice, 31.1 mg/kg (range, 26.8 to 39.0 mg/kg); Sprague Dawley male mice, 26.5 mg/kg (range, 22.5 to 31.2 mg/kg).

Intraperitoneal doses of SA into male CD1 mice caused pulmonary atelectasis and focal peritonitis involving capsular surfaces of kidney and liver with subjacent hepatic parenchymal necrosis. Death by cardiac or pulmonary insufficiency or both was suggested by the pulmonary hem-

TABLE 5. Production of SA *P. oxalicum* NRRL 5209 on YES medium^a

Day	Still culture (g/liter)	Shake culture (g/liter)
7	1.4	0.01
9	2.0	0.20
15	1.9	0.04
17	2.8	—
20	2.4	—
22	1.2	—

^a 500 ml/Fernbach; 28°C incubation. No SA D was detected at 37°C in either still or shake culture.

^b —, Nondetectable.

orrhages and massive atrial dilation in mice given 30 mg or more of SA per kg.

No deaths or gross teratogenic effects occurred in chicken embryos at doses up to 150 µg/embryo, the upper limit of solubility for the toxin in cottonseed oil or NaHCO₃.

SA D is a poor antibiotic with only very slight activity against *Micrococcus luteus* and *Bacillus subtilis* and no activity versus *Candida albicans*, *Saccharomyces cerevisiae*, *Escherichia coli*, *Mucor rhomania nus*, and *Mycobacterium phlei*.

DISCUSSION

The fungus *P. oxalicum* commonly found on cereals was reported by Mislivec and Tuite (7) to be the dominant *Penicillium* on freshly harvested corn kernels. In a survey of toxigenic fungi isolated from cereals in South Africa, Scott (8) reported *P. oxalicum* to be toxic to ducklings. Steyn (9) subsequently reported SA D as the major toxin produced by this fungus. The same compound was isolated by Andersen et al. (1) from *Aspergillus aculeatus* cultured on corn.

P. oxalicum is a common contaminant of major commodities, and all the strains we examined produced SA. We infer that the possible importance of SA as a mycotoxin of economic significance may have been underestimated. This situation may be a result of many reports on toxic secondary metabolites produced by fungi to which no mycotoxicosis have been attributed and the publication by Yamazaki et al. (12) in which SA A was reported to be nontoxic to chicken embryos. We obtained similar negative data for chicken embryos, possibly because the toxin may be primarily a lung toxicant and, hence, should show no activity in embryonic systems.

The mycotoxicoses that involved massive lung edema and necrosis of the cerebrum in Louisiana horses may have been the result of a mixed mycotoxicosis because the overt symptoms demonstrated for *F. moniliforme*-induced leukoencephalomalacia in donkeys (11) and for SA in

laboratory animals (C. S. Reddy, A. W. Hayes, W. L. Williams, and A. Ciegler, Toxicol. Appl. Pharmacol., in press).

We attempted to repeat the mycotoxicosis in pigs with the corn originally implicated in the Louisiana outbreak, but no toxic effects were observed. The corn was about 1 year old when the experiment was attempted; hence, negative results possibly could be attributed to toxin deterioration.

The high yields of SA obtained on various grains by fermentation with *P. oxalicum* (up to 3 g of toxin per kg of grain) are indicative of the potential hazard of this commonly occurring fungus. Additionally, its ability to grow profusely at 37°C with abundant conidial formation should also cause concern as a potential pulmonary intoxicant.

The finding of *P. oxalicum* in prairie grasses suggests that these grasses and possibly other wild vegetation may serve as a source of contaminating inoculum for row crops. This complex concept of an ecological relationship between row crops, mycotoxin-producing fungi, and the environment is a new broad research area.

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