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Purification and Partial Characterization of Flavotoxin A

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A heat-resistant, low-molecular-weight toxin was isolated from semisolid potato dextrose agar medium after inoculation with *Flavobacterium farinofermentans* sp. nov., which was isolated from fermented corn meal that caused some outbreaks of food poisoning in China. The toxin was purified by solvent partition, Sephadex LH-20 gel filtration, and C-18 reversed-phase column chromatography. Thin-layer chromatography and high-pressure liquid chromatographic methods were developed for the identification and analysis of the toxin. The purified toxin exhibited a single spot in thin-layer chromatography and a single peak in high-pressure liquid chromatography and had adsorption maxima at 232 and 267 nm. Mass spectral analysis indicated a molecular weight of 169 with an experimental formula of $C_9H_{13}O_3$. The 50% lethal dose of purified toxin in mice (oral) was less than 6.84 mg/kg, but greater than 0.68 mg/kg. Postmortem examination showed that the mice died of some type of neurological and cardiovascular system toxicity. The name Flavotoxin A is being assigned to the toxin.

Food poisoning caused by the consumption of fermented corn meal has been reported occasionally in certain regions of China. The incidents were more concentrated in northeastern China, where people consume foods such as corn bread, noodles, and dumplings prepared from the fermented corn meal. The fermented corn meal is generally prepared by soaking corn in water at room temperature for 2 to 4 weeks, washing with water, and finally grinding the wet corn into flour for subsequent uses. The outbreaks generally occurred between July and September and occasionally occurred in May and October. Between 1961 and 1979, 23 outbreaks involving 327 persons were reported. All of those involved had consumed foods prepared from fermented meal, and 314 persons were poisoned within 1 to 10 h after consuming the foods. A high mortality rate (101 persons dead among 314 poisoning cases, or 32.2%) was documented (5).

In 1961, King (1) isolated a strain of bacteria from toxic fermented corn meal and found that the organism produced yellow pigment in culture. Mice fed the fermented meal that had been inoculated with the bacteria died with symptoms similar to those observed in human intoxication. Subsequent studies by the Research Group for Pathogenesis of Fermented Corn Flour Poisoning of China (5) led to the conclusion that the poison was, indeed, caused by the bacteria. They named the new species *Flavobacterium farinofermentans* sp. nov. (5). Forty strains of the species were isolated from the toxic meal, and their toxicity was tested. After inoculation on either the fermented corn meal or potato dextrose agar, the bacteria produced a heat-stable, low-molecular-weight exotoxin that produced toxic symptoms and pathological alterations in mice, dogs, and monkeys similar to those observed in humans. Methods for the production and partial purification of this new toxin were also established by the research group. In the present collaborative study, a method for the purification of this toxin was established, and some properties of the purified toxin were investigated. Details for the purification, characterization, and analysis of this toxin are described in this paper. For the sake of description, we tentatively name the new toxin Flavotoxin A.

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MATERIALS AND METHODS

Materials. The crude toxin was prepared at the Institute of Health, Chinese National Center for Preventive Medicine, Beijing, China, by the procedure of Meng et al. (4). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). C-18 reversed-phase silica gel (LiChroprep RP-18; particle size, 25 to 40 μM) was an E. Merck product (Darmstadt, Germany) and was obtained from American Scientific Products (McGaw Park, Ill.). Precoated thin-layer chromatography (TLC) plates (KC-18F) were obtained from Whatman Ltd. (Clifton, N.J.). Solvents for high-pressure liquid chromatography (HPLC) were obtained from Burdick & Jackson Laboratory, Inc. (Muskegon, Mich.) and were of HPLC grade. Other solvents and chemicals were either reagent grade or chemically pure. CF-1 mice were obtained from Harlan Sprague-Dawley Laboratory, Inc. (Madison, Wis.).

Production and partial purification of the toxin. For toxin production and preparation of crude toxin, the method of Meng et al. (4) was followed. Briefly, the crude toxin was produced in a semisolid potato dextrose agar plate covered with sterilized cellophane and inoculated with 0.5 ml of stock *F. farinofermentans* sp. nov. culture that had been grown in Sabouraud media for 24 h at 37°C. After incubation at 26°C for 5 days, the cellophane was removed, and the plate was heated at 100°C for 30 min and then placed in a freezer (−20 to −60°C) for 48 h. After thawing, the liquid fraction was collected and concentrated to ca. two-thirds of its original volume in a rotary evaporator. The concentrate was cooled in a refrigerator overnight and filtered. The filtrate was concentrated again to remove the water and precipitated first with methanol (MeOH) and then with acetone. The toxin was located in the sediment, which was then dehydrated with ethanol. The residue of this ethanolic precipitation, which contained all of the toxic activity, was used in the following purification.

Purification steps. (i) Sephadex LH-20 gel filtration. In a typical experiment, 0.45 g of the crude concentrates obtained from the last step were dissolved in 2.5 ml of 10% MeOH in water and then passed through a Sephadex LH-20 column (3.5 by 35 cm) that had been equilibrated with MeOH-chloroform (1:1). The column was then washed with

MeOH-chloroform (1:1), and the absorbances of the eluents were monitored at 254 nm with a model UV-4 absorbance monitor (Instrumentation Specialities Co., Lincoln, Neb.). The flow rate of the chromatography was controlled by a Milton-Roy minipump (model 196-0065-002; Laboratory Data Control, Riviera Beach, Fla.). TLC and HPLC were used to examine the effectiveness of the separation. In an initial study, the eluents obtained from the column were divided into eight fractions, and the toxicity of each fraction was tested. The materials containing toxin were pooled and concentrated to near dryness by rotatory evaporation.

(ii) C-18 reversed-phase column chromatography. The toxin-containing material obtained from the Sephadex column was redissolved in 2 ml of 10% MeOH-water solution and passed through a C-18 reversed-phase column (1 by 25 cm) equilibrated with MeOH-water (25:75). The column was first washed with 50 ml of 25% MeOH containing 0.5% glacial acetic acid (HOAc), then with 50 ml of 50% MeOH containing 0.5% HOAc, and finally with ca. 300 ml of 70% MeOH containing 0.5% HOAc. Starting from the elution of the last solvent system, the eluent was collected in 3.39-ml fractions with a fraction collector. The flow rate for chromatography was controlled by a Milton-Roy minipump at 1.5 ml/min.

Toxicity test. To monitor the toxicity obtained from each fraction during the purification, strain CF-1 mice were used. Each mouse (18 to 21 g) was dosed with 0.5 ml of solution via a stomach tube. The toxin was diluted with 0.85% saline immediately before dosing. At least four mice were used for each toxin level or each fraction obtained from the column chromatography. The number of mice that died and the time of death was recorded. In addition, both the dead and healthy mice were examined for gross pathological changes after the administration of the toxin.

Analyses. (i) TLC. TLC analysis was carried out on a precoated reversed-phase TLC plate (0.2 mm) with fluorescence indicator and developed in a solvent system of MeOH-water-HOAc (70:30:0.5). After development the plate was observed under both long and short UV. Occasionally, the plates were charred with 10% H_2SO_4 in ethanol.

(ii) HPLC. HPLC was carried out on a model ALC 202 liquid chromatographic system equipped with an A6000 pump, a U6K septumless injector, and a model 720 system controller (Waters Associates Inc., Milford, Mass. and a variable-wavelength UV detector (Laboratory Data Control) set at 254 nm. A μ-Bondapak C18 reversed-phase Radial-Pak cartridge column in conjunction with a Z-module radial compressor (Waters Associates) was used for all of the separations and analyses. A solvent system of MeOH-water-HOAc (75:25:1.7) was used throughout the experient. The chromatography was carried at a flow rate of 2.0 ml/min. Flavotoxin A was eluted from the column at 10.8 min.

(iii) Spectrophotometric. UV spectra were determined in a Beckman model HS spectrophotometer with a light path of 1 cm. Mass spectra were obtained on an AEI model MS-9 mass spectrometer (Associated Electronics Inc., United Kingdom).

RESULTS

Preliminary identification of toxic component. In the first preliminary study, 0.45 g of crude preparation in 2.5 ml of 10% MeOH-water solution was applied to the Sephadex LH-20 column. The eluents were divided into eight fractions according to their UV absorption. The materials in each fraction were concentrated and redissolved in 60% MeOH-water solution. A sample from each fraction was further diluted with saline to a final concentration equivalent to 4.0 mg of original crude material per ml of saline, of which 0.5 ml was fed to each mouse. Results showed that the toxin was located in the last fraction (tubes 70 through 180, elution volume between 700 and 1,800 ml). Among four mice tested, two mice died at 127 min, one died at 149 min, and one died at 163 min after intubation of the toxin. No toxic effect was demonstrated in other fractions. The preparation showed one major strong dark spot (R_f of 0.40) in TLC and two other, minor spots under short-UV light. Under long-UV light, two minor, weakly fluorescent spots (R_f of 0.64 and 0.72) were seen. The R_f 0.40 spot was identified as flavotoxin A.

In the second preliminary experiment, materials obtained from the Sephadex column were subjected to a preparative TLC. A total of eight bands with or without fluorescence was observed. The different bands were scraped from the plate and eluted with methanol. Material obtained from each band was again tested for its toxicity. The toxin was found exclusively in the strong dark band, which had an R_f of 0.40 in TLC.

In the third preliminary experiment, materials obtained from the preparative TLC were subjected to the C-18 reversed-phase column. We found that the toxin can only be eluted from the column with 70% aqueous MeOH containing 0.5% HOAc. The material obtained from the column gave a single spot in TLC and one peak in HPLC. After the solvent was removed by rotatory evaporation, the materials were dried under vacuum. This preparation was used as a preliminary toxin standard in HPLC for subsequent detailed studies.

Sephadex LH-20 filtration. After establishment of the HPLC method for analysis of the toxin, the distribution of toxin in each fraction was determined. A typical elution pattern for the isolation of flavotoxin A by Sephadex gel filtration is shown in Fig. 1. In this experiment, 0.45 g of crude toxin containing 35.6 mg of flavotoxin, as analyzed by HPLC, was applied to the column. Two major peaks were obtained. HPLC revealed that all of the toxin was confined in the second peak (tubes 70 through 180, 700 to 1,800 ml). The materials in the second peak were divided into three

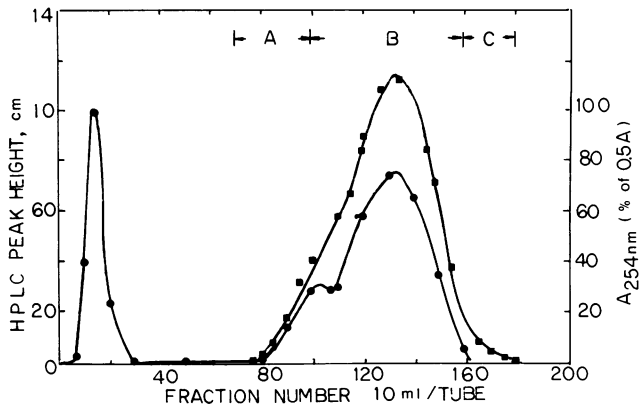

FIG. 1. Chromatography of crude flavotoxin A in Sephadex LH-20. The elution pattern was obtained from a run in which 0.45 g of crude toxin was added to a column (3.5 by 35 cm) that was equilibrated and developed with chloroform-MeOH (1:1). The flow rate was 0.71 ml/min. The absorption at 254 nm (●) was measured by a flow cell, and the maximum absorbance for the monitor was set at 0.5. The relative concentration of toxin (■), as determined by HPLC analysis, is expressed as centimeters of peak height of the elution peak containing toxin (Fig. 3) rather than as absolute values.

fractions, and the toxin content in each fraction was determined by HPLC. A total of 3.6 mg (10.2%), 28.9 mg (81.2%), and 2.1 mg (5.9%) of the toxin added to the column was recovered in fractions A (tubes 70 through 100, 700 to 1,000 ml), B (tubes 101 through 160, 1,010 to 1,600 ml), and C (tubes 161 through 180, 1,610 to 1,800 ml), respectively. The materials in fraction B showed a small peak, which had a retention time of 14.5 min, in addition to flavotoxin A, which had a retention time of 10.8 min. TLC analysis also showed additional spots other than flavotoxin A.

Chromatography of partially purified flavotoxin A on C-18 reversed-phase silica gel column. The materials obtained from the Sephadex column gel filtration fraction B were concentrated, dissolved in 2 ml of 25% MeOH in water, and then loaded onto the C-18 reversed-phase column. After appropriate washings, the column was eluted with 70% MeOH containing 0.5% HOAc. A typical elution pattern is shown in Fig. 2. Flavotoxin A was eluted from the column between 100 and 165 ml after starting elution with the MeOH-water-HOAc (70:30:0.5) solvent. Materials in tubes 1 through 34, 35 through 37, and 38 through 53 were pooled as fractions A, B, and C, respectively. A total of 2.65 mg (9.2%), 19.61 mg (67.8%), and 4.28 mg (14.8%) of toxin was recovered in fractions A, B, and C, respectively. The toxin obtained from fraction B was essentially chromatographically pure. HPLC and TLC analysis of fraction B revealed no additional peaks or spots.

Analyses. TLC analysis showed that the purified toxic material exhibited one strong dark spot with an R_f value of 0.4 under short-UV light. HPLC showed only a single peak (Fig. 3) that had a retention time of 10.8 min. When the toxin solution (in MeOH) was kept in the refrigerator for more than 3 days, two spots with R_f values of 0.43 and 0.18 were observed. However, this solution still exhibited a single peak in the HPLC. The purified toxin absorbed maximally at 232 and 267 nm. High-resolution mass spectrum analysis revealed a base peak at an *m/e* of 169 (Fig. 4) with an

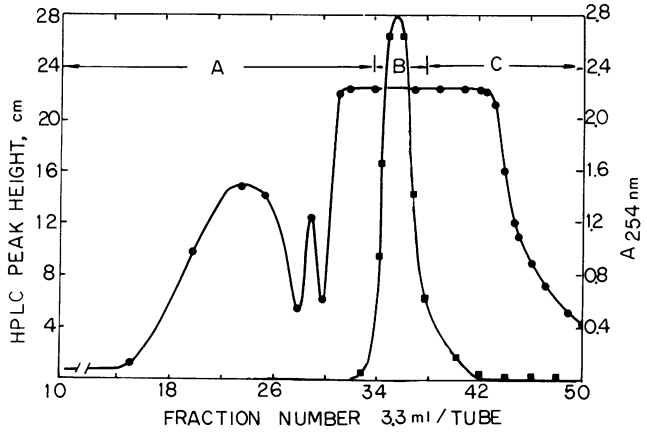

FIG. 2. Chromatography of partially pure flavotoxin A in a C-18 reversed-phase column. The elution pattern was obtained from a run in which the partially purified toxin obtained from the Sephadex column was applied to a C-18 reversed-phase column (1 by 25 cm) that was equilibrated with MeOH-water (25:75). After washing with appropriate solvents containing different concentrations of MeOH (see the text), the column was developed with MeOH-water-HOAc (70:30:0.5), and all material were collected in a fraction collector. The flow rate was 1.5 ml/min. Again, the absorbance at 254 nm and the relative toxin concentration were determined as described in the legend to Fig. 1.

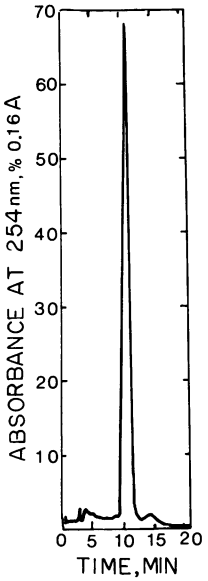

FIG. 3. HPLC of flavotoxin A.

experimental formula tentatively assigned as $C_9H_{13}O_3$. No molecular ion was found. The largest fragment at an *m/e* of 44 was primarily due to the CO_2 fragment. Nuclear magnetic resonance analysis was unsuccessful.

Toxicity test. In a feeding study, each of four mice, weighing an average of 19 g, were fed with 0.13 mg of the purified toxin. All mice died between 1.5 and 2.5 h after feeding. However, the mice survived when fed by intubation at a dosage of 0.013 mg of the purified toxin. Since the average weight of the mice was 19 grams, the 50% lethal dose should be less than 6.84 mg/kg, but greater than 0.68 mg/kg. In addition to the recording of the death rate and time of death after feeding, four mice were opened surgically, and a gross pathological examination was performed on each animal immediately after death.

As death approached, the animals developed marked respiratory irregularities. First, rapid breathing developed. This led to rapid, gasping breathing with periods of respiratory cessation. Eventually, the respiration stopped, with death ensuing.

As the breathing difficulties developed, the animals were lethargic and inactive. Soon, and as death approached, the animals showed minor disturbances and balance upsets, with

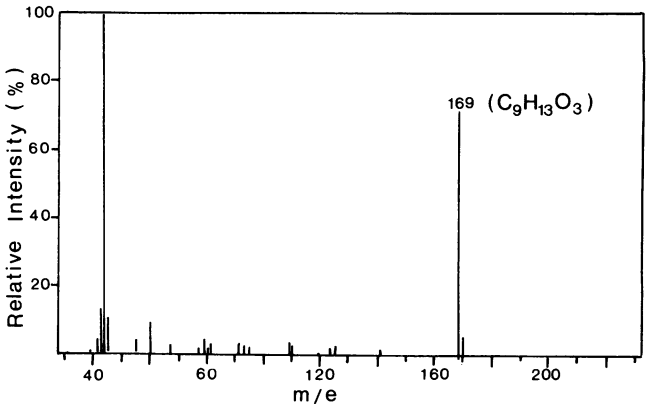

FIG. 4. Mass spectrum of flavotoxin A.

eventual rigid paralysis of the extremities, especially the hindquarters.

Two of the mice showed evidence of pulmonary edema and congestion upon gross dissection. All of the other organ systems appeared normal and apparently unaffected by the purified toxin. Thus, the toxin has some neurological as well as cardiovascular system toxicity.

DISCUSSION

In the present study, we demonstrated that the cause of food poisoning outbreaks in certain regions of China resulted from the ingestion of fermented corn meal containing a heat-resistant, low-molecular-weight toxin produced by *F. farinofermentans* sp. nov. The toxin can readily be produced in PDA agar and can subsequently be isolated in pure form by solvent partition, Sephadex gel filtration, and C-18 reversed-phase column chromatography. It can also be analyzed by an HPLC procedure. Preliminary studies indicated that the toxin is a low-molecular-weight, organic compound. The cause of death by this new toxin appears to be due to neurological and cardiovascular blockage. Because the mass spectrum for this toxin was very simple and also the major fragment was CO_2, we suspect that the observed base peak might arise from a larger-molecular-weight compound. The simplicity of the mass spectral character is very similar to Bongkrekic acid (molecular weight 486, $C_{28}H_{38}O_7$), for which a base peak at an *m/e* of 183, i.e., 169 + 14 (methyl group), was observed for the methylated compound (3). Bongkrekic acid is a branched, unsaturated tricarboxylic acid and is produced by *Pseudomonas cocovenenans*, an organism similar to the *Flavobacterium* spp. (6). It has been found to be the major toxin involved in the Bongkrek food poisoning (6). Like Bongkrekic acid (2), flavotoxin A has acid characteristics and absorbs maximally at 232 and 267 nm (versus 239 and 267 nm for Bongkrekic acid). The determination of whether these two toxins are structurally and biosynthetically related requires additional studies. With the establishment of a methodology for isolation and analysis of the toxin, future work should be directed to the structural identification and pharamacology of the toxin. Furthermore, the etiology for toxin production and methods for detoxification warrant additional investigation.

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