Purification and Properties of a Toxin Isolated from *Mortierella wolffi*

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Received for publication 14 August 1973

A substance toxic for mice has been extracted from the washed mycelia of *Mortierella wolffi*. This toxin could also be demonstrated in culture fluids after spontaneous autolysis of the fungal hyphae. Purification of the toxin was carried out using Sephadex chromatography (G-200, G-50) and resulted in a single band on sodium dodecyl sulfate polyacrylamide gels. Properties of the toxin included heat lability at 60°C, trypsin sensitivity, activity over a wide pH range, and a molecular weight of 23,600. The mean lethal dose for 16-week-old male mice was 3.3 μg of protein.

Mucormycosis is an opportunistic fungal infection which usually occurs in the presence of lowered or altered host resistance and which may affect any organ of the body. Various species of *Absidia, Mucor,* and *Rhizopus* were incriminated in the past as the causative agents (3). Recent investigations (1) in which histological observations were supported by cultural studies showed that members of the *Mucorales,* and in particular the genus *Mortierella* (e.g., *Mortierella wolffi*), may be of considerable importance. Apart from the fact that numerous phycomyces all appear to show a predilection for growth within blood vessels, little is known of the pathogenesis of the disease.

This study was undertaken in an attempt to determine if toxins produced during the growth of *M. wolffi* could be incriminated in the disease process, and, if so, to investigate the properties of the toxin(s).

**MATERIALS AND METHODS**

**Culture.** A strain of *M. wolffi* (68/4535) obtained from a bovine placenta was used in all experiments.

**Preparation of spore suspension.** *M. wolffi* spores were obtained from 10-day-old cultures grown on hay agar (3 g of finely chopped hay in 1,000 ml of distilled water containing 2% agar) at 30°C, by washing the cultures with sterile phosphate-buffered saline (PBS, pH 7.2) containing 1% Tween 80. The suspension was then centrifuged, and the supernatant fluid was decanted. After being washed three times, the spores were resuspended in PBS containing Tween 80 and stored in 1-ml amounts at -20°C until required.

**Growth of *M. wolffi* in liquid media.** Approximately 400 spores were inoculated into 32 screw-capped bottles containing 4 ml of glucose (4%)-neopeptone (1% Difco) broth. The spores were then incubated at 37°C as stationary cultures. The time period of the experiment was 168 h. At 24-h intervals, the medium was decanted from four samples and the fungal deposits were "killed" by addition of 1% Formalin. After washing and resuspending the deposits in distilled water, the amount of fungal growth was determined by lyophilization to a constant weight, over phosphorus pentoxide. The supernatant from each sample was pooled and transferred to dialysis tubing and concentrated to approximately 2 ml with Carbowax. The toxicity of concentrated supernatants was determined by inoculation of 0.2-ml amounts into 3 pairs of mice (see below).

**Preparation of *M. wolffi* toxin.** The fungus was inoculated in Roux bottles containing 200 ml of glucose-neopeptone broth. After 14 days of incubation at 37°C, the fluid was decanted from each bottle and the mycelial mats were washed with sterile saline. The mats were then ground in 10 ml of saline with a mortar and pestle containing sterile sand for 5 min for each of 3 consecutive days. Between grindings, the materials were kept at 4°C. The ground mycelium was then centrifuged, and the supernatants were placed in dialysis tubing and concentrated 10-fold with Carbowax. After filtration through a 0.45-μm membrane filter (Millipore Corp.), the concentrated mycelial extract was stored at -20°C.

**Purification of toxin.** A 3-ml amount of the concentrated mycelial extract was placed on a Sephadex G-200 (Pharmacia Fine Chemicals) column (1.5 by 90 cm) equilibrated with 0.1 M phosphate buffer (pH 7.0). Elution was carried out with the same buffer at a flow rate of 11 ml/h. The eluate was monitored at 254 nm by use of an LKB Uvicord flow cell, and 3-ml fractions were collected and assayed for toxicity by mouse inoculation (see below). Toxic fractions were rechromatographed on another Sephadex G-50 column (1.5 by 90 cm), the elution and toxin assay being carried out as before. Toxic fractions resulting from this step were finally passed through a G-50 Sephadex column (1.5 by 30 cm), and the toxic fractions were stored at 4°C.
Toxin assay. Male mice approximately 16 weeks old were used in all experiments. The mean lethal dose (LD₅₀) value was calculated by preparing twofold dilutions of the toxin in phosphate buffer (pH 7.0). Three mice were used for each dilution. The animals were inoculated through a lateral tail vein with 0.2 ml of each dilution and observed for death over a period of 4 days.

Protein determination of toxin. The protein estimation of the toxic mycelial extracts was determined by the method of Lowry et al. (5) using crystalline bovine serum albumin (Sigma) as the standard.

Polyacrylamide gel electrophoresis of toxin. (i) The polyacrylamide gel electrophoresis technique used was essentially that of Davis (2). Four percent (wt/vol) polyacrylamide gels were prepared in glass tubes (7 by 75 mm) in 0.37 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer. Samples (50 aliters) containing approximately 20 μg of protein were applied to the top of the gels and overlaid with reservoir buffer. Electrophoresis was carried out at 5 mA/gel for 30 min. The gels were then removed from the tubes, fixed in 20% sulfosalicylic acid, stained in 0.25% Coomassie blue for 2 to 3 h, and destained electrophoretically or by washing in repeated changes of 7% acetic acid.

(ii) Sodium dodecyl sulfate (SDS) polyacrylamide gels were prepared in 0.1 M phosphate buffer (pH 7.8) with 0.1% SDS. Samples were subjected to electrophoresis for 5 h at 8 mA/tube after a period of 20 min at 2 mA/tube. Gels were then fixed and stained as above.

Elution of protein from acrylamide gels. Unstained acrylamide gels, without SDS, were sliced and then eluted with 0.1 M phosphate buffer (pH 7.0) at 37 C for 48 h. Concentration was carried out in dialysis tubing by use of Carbowax. Fractions were inoculated into mice, and the animals were observed for death over a period of 10 days.

Characterization of the toxin. The effect of (i) heating for 60 min at temperatures between 50 and 65 C, (ii) pH ranges of 2.9 to 9.2, and (iii) trypsin on the toxicity of the mycelial extract was determined by inoculating mice with suitably treated samples.

Molecular weight estimation. The molecular weight of the toxin was calculated by use of SDS polyacrylamide gels and known marker proteins. The marker proteins consisted of bovine serum albumin fraction V, carboxypeptidase A, egg albumin, cytochrome c, and pepsin. The procedure was similar to that already described for SDS polyacrylamide gels using 20 μg of marker proteins and toxin. All gels were measured before electrophoresis and after fixation and staining. Distances migrated were measured from the center of the band to the top of the gel. By regression analysis the “best fit” line for the points was calculated.

Molecular weight was also determined by Sephadex chromatography by use of a G-200 column. The distribution coefficient, Kav, of the toxin was determined by using Sephadex standards.

RESULTS

Growth rate of M. wolfii in liquid medium. The growth rate of M. wolfii in glucose-neopeptide broth is shown in Fig. 1. Maximum growth as measured by mycelial mass was reached at approximately 96 h. Toxic activity could not be detected in the supernatant fluid before 96 h. By 120 h, at which time autolysis of the hyphal elements was well advanced, the supernatant possessed toxic properties.

Sephadex filtration. After initial extraction of the ground mycelium the supernatant had an opalescent appearance with a darkish brown pigmentation.

The elution profile of the crude extract from Sephadex G-200 gel filtration is shown in Fig. 2A. Toxic activity in mice was observed only in fractions 31 to 37 inclusive. Figure 2B shows the elution pattern when fractions 31 to 37 were rechromatographed on Sephadex G-50; toxicity being observed in fractions 19 to 21. Finally, when these fractions were run through a G-50 column, only one peak was obtained (Fig. 2C) which was shown to be toxic.

Polyacrylamide gel electrophoresis. The various stages of purification of the toxin are shown in Fig. 3. When purified toxin from the final G-50 chromatography was run on SDS polyacrylamide gels, only one band was obtained (Fig. 3D). This was interpreted to mean that the toxin was relatively pure. The molecular weight of the toxin was estimated to be 23,600 ± 1,000 as judged by the mobility of the molecular weight markers is SDS polyacrylamide gels. Chromatography in Sephadex G-200 gave a Kav value of 0.5 from which a molecular weight of 24,000 ± 2,000 was estimated. On polyacrylamide gels without SDS, aggregates were formed as shown in Fig. 3E. Material eluted from the single band (T) was found to kill mice in 2 days. The other bands appear to be aggregates of band T. When the distances

FIG. 1. Growth curve of M. wolfii in neopeptone-glucose broth. Dry weight of mycelial mats was determined after lyophilization, and the toxicity of concentrated supernatants was determined by inoculation of 0.2 ml into mice. Shaded area shows toxicity.
migrated were plotted against the log of the molecular weights (Fig. 4), a straight line was obtained. When these aggregates were eluted and rerun on another gel, band T was predominantly obtained.

Fig. 2. Purification of M. wolfii toxin by Sephadex gel filtration. A, Profile obtained from Sephadex G-200 column (1.5 by 90 cm). B, Rechromatography of toxic fractions from A, on Sephadex G-50 (1.5 by 90 cm). C, Final purification step on a Sephadex column (1.5 by 30 cm). Fractions (3 ml) were collected and toxicity was measured in mice.

Fig. 3. SDS polyacrylamide showing the various stages of purification of M. wolfii toxin. A, crude extract; B, after G-200 Sephadex chromatography; C, after G-50 Sephadex chromatography; D, final purification step after G-50 chromatography. Gel E shows the aggregates of toxin in polyacrylamide gels without SDS. Band T was found to be toxic when eluted and assayed in mice. Note: the bottom of the gel was broken during preparation.

Properties of M. wolfii toxin. Before death, mice appeared lethargic but could be “excited” by noise. At autopsy the only consistent gross abnormalities were constriction of the intestines and enlarged, pale-grey kidneys. On rare occasions, intestinal, hepatic, or pulmonary hemorrhage was seen. Histological examination of affected kidneys revealed marked necrosis of the tubular epithelium (unpublished results). The following effects were noted. (i) Effect of heat: no deaths were observed in mice inoculated with toxin which had been heated to 60°C for 60 min. (ii) Effect of pH: the toxin was active over the entire pH range 2.6 to 9.2. In all cases inoculated mice died within 24 h. (iii) Effect of trypsin: trypsin digestion destroyed the activity of the toxin. Inoculation of a trypsin solution had no effect on mice. (iv) The LD₅₀ value for 16-week-old mice was 3.3 μg of protein.

DISCUSSION

The studies presented here demonstrate the presence of toxin in cultures of M. wolfii. Most of the toxic activity was associated with the fungal hyphae and could be detected in the growth medium only when lysis of the mycelium was well advanced. Purification procedures on
the crude hyphal extract resulted in an increase in specific activity of 180-fold. Elution through a Sephadex G-200 column eliminated most of the contaminating material, and subsequent, rechromatography of the toxic fractions on G-50 columns resulted in a relatively pure form of the toxin as judged by the formation of a single protein band after SDS polyacrylamide gel electrophoresis. Unlike many proteins which tend to form oligomers during the purification process, this was not a problem with the toxin. The aggregates were readily disrupted with SDS. While elution from gels of the single unstained band proved to be toxic when inoculated into mice, the aggregates were not toxic to mice, but this could be due to an insufficient amount of toxin.

The properties of heat lability and trypsin sensitivity would strongly suggest that the toxic molecule is a protein. However, stability over a wide pH range is an unusual feature and is shown by only a few proteins, e.g., interferon (4) and hepatitis B antigen. Furthermore, the toxin either as a crude extract or as purified material may be stored at -20 C for at least 4 months without any apparent loss of activity. Other features noted during the investigation were that activity was not affected by Seitz filtration at pH 7, by dialysis, by freezing and thawing, or by lyophilization. The molecular weight of

23,600 obtained on polyacrylamide gels is in good agreement with the molecular weight (24,000) obtained by Sephadex filtration.

Other fungi from which a similar toxin has been recovered are Aspergillus fumigatus and Aspergillus flavus (8). These toxins were similar to the toxin isolated from M. wolfii in the properties of heat lability, trypsin sensitivity, activity over a wide pH range, and renal involvement in experimental animals.

Substances liberated from live or degenerating fungal elements can possibly be incriminated in the pathogenesis of a few mycoses. The tuberculcule-like lesions characteristic of avian pulmonary aspergillosis may possibly result from toxic substances, or host reaction to nontoxic substances, liberated from the central hyphal mass. It was suggested (6) that tissue damage occurring in this manner could lead to further fungal invasion and the "centrifugal" spread of the lesion. The amorphous eosinophilic hyphal sleeves seen in Basidiobolus haptosporas and Entomophthora coronata infections seem to result from host hypersensitivity to hyphal components (9), whereas hyphal metabolites are possibly of significance in determining the appearance and duration of dermatophyte skin lesions (7). Toxin-like substances produced during the uncontrolled growth of Candida albicans on mucous membranes may induce local tissue necrosis and a subsequent invasion of the dead tissue by hyphae (10). The biological significance of the toxin from M. wolfii is at present under investigation.

ACKNOWLEDGMENTS

We wish to thank J. A. R. Miles, Department of Microbiology, University of Otago, Dunedin, New Zealand, for providing facilities to undertake this research which was supported in part by the Medical Research Council of New Zealand. M. E. di Menna, Ruakura Agricultural Research Centre, kindly supplied the strain of Mortierella wolfii used.

LITERATURE CITED