Use of thin layer chromatography for detection and high performance liquid chromatography for quantitating gliotoxin from rice cultures of *Aspergillus fumigatus* Fresenius

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Abstract

Gliotoxin, a mycotoxin with antimicrobial and immunosuppressive capabilities, is produced by several genera of fungi including the pathogenic fungus *Aspergillus fumigatus*. The ability of selected isolates of *A. fumigatus* to produce gliotoxin on three different media was tested and a thin layer chromatographic and high performance liquid chromatographic method for quantitation of gliotoxin from rice culture was developed and is described. Rice cultures were extracted with chloroform and the resulting extract was partially purified by precipitation with petroleum ether and cleanup by gel permeation chromatography. Gliotoxin was detected by thin layer chromatography and quantitated by high performance liquid chromatography using a U.V. absorbance detector with a 254 nm filter and a mobile phase of methanol-water 43:57 (V/V) with a flow rate of 2.0 ml/min. The retention time for gliotoxin was approximately 4.8 min. From rice samples spiked with gliotoxin concentrations of 0.67, 1.33, 2.67, 4.00 and 5.33 μg/g the average recovery was 83.8%.

Introduction

Gliotoxin (Fig. 1) is a fungal toxin belonging to the class of fungal metabolites called epipolythiodioxopiperazines [10, 24]; some mycotoxins in this class cause economically important diseases of livestock [14]. Gliotoxin was discovered in 1936 by Weindling [27] and since then has been isolated from at least five genera of fungi including six species of *Aspergillus* and *Penicillium* [10, 16].

Gliotoxin has a number of biological properties including inhibition of fungal [9] and bacterial [1] growth, extending the growth lag phase of *B. subtilis* [3], and antiviral activity [11]. Murine lymphosarcoma cells exposed to gliotoxin and then transplanted into recipient mice failed to grow [13]. Additionally, in *in vitro* tests, gliotoxin inhibited (a) phagocytosis by rodent macrophages; (b) mitogenic stimulation of lymphocytes; and (c) induction of alloreactive and MHC restricted cytotoxic T cells [15]. The compound also caused fragmentation of spleen cell DNA [2] and modified the DNA of *B. subtilis* as measured by a “Rec” assay for genotoxicity [1].

Among gliotoxin producing fungi is *A. fumigatus* Fresenius, the predominant pathogen of aspergillosis in man and other animals. Avian aspergillosis is an economically important disease for poultry farmers while aspergillosis is a major disease in human patients whose cell-mediated immunity has been altered by drugs or is defective from other causes [19]. A major first line of cellular defense against aspergillosis is the alveolar
macrophage that is capable of ingesting and killing inhaled conidia of *A. fumigatus* [12, 20, 21]. Gliotoxin [15] and other mycotoxins, such as aflatoxin and T-2 toxin [17, 18], inhibit phagocytosis by macrophages. Although the involvement of gliotoxin in the pathogenesis of aspergillosis has not been demonstrated, Eichner and Mullbacher [6] have hypothesized that gliotoxin may be produced during the pathogenic state of *A. fumigatus* and contributes to the pathogenicity of this fungus. Also, it is unknown whether gliotoxin occurs naturally in commodities. To assess the occurrence of gliotoxin in commodities or perhaps animal tissues, a sensitive analytical method is required.

Gliotoxin has been detected from fungal cultures by thin-layer chromatography [26], but because visualization of gliotoxin was dependent upon fluorescence quenching, the sensitivity of the test was limited. We determined that nine pathogenic strains of *A. fumigatus* produced gliotoxin and then maximized the conditions for production on rice by one of these isolates. Also we developed a thin layer chromatographic method and reverse phase high performance liquid chromatographic (HPLC) method that provides a reliable, quantitative analysis of gliotoxin in a commodity.

**Materials and methods**

**Gliotoxin standards.** Powdered gliotoxin standard was a generous gift from Richard J. Cole (National Peanut Research Lab., Dawson, GA 31742) and Rex T. Gallagher (Ruakura Animal Research Station, Hamilton, New Zealand). Standards were kept in foil-covered containers at −70 °C.

**Organisms.** Nine pathogenic isolates of *Aspergillus fumigatus* were selected to determine their ability to produce gliotoxin. These isolates and their sources are listed in Table 1.

**Screening of Aspergillus fumigatus isolates for gliotoxin production**

Each of the nine isolates of *A. fumigatus* were grown in three flasks of each of three different media. Eagle's F-15 medium flasks were prepared by placing 100 ml sterile Eagle's medium (Grand Island Biological Co., Grand Island, NY) with 5% fetal calf serum in each of 30 sterile 300 ml cotton-stoppered Erlenmeyer flasks. The same number and size of flasks were prepared containing YES medium [5]. Rice medium was prepared as described [22] using 1-L cotton-stoppered Erlenmeyer flasks. Triplicate cultures of each media were inoculated with a loopful of conidia from a one-week-old Sabouraud dextrose agar slant culture of each test organism. Uninoculated flasks of each medium served as controls. The two liquid media were incubated as stationary cultures for 6 days. Eagle's medium cultures were incubated at 37 °C and the YES medium cultures were incubated at 28 °C. The rice cultures were placed on an incubator shaker at 300 rpm and 28 °C.

The liquid cultures were harvested by filtering through Whatman No. 1 filter paper and the filtrate from the three flasks of each medium of each test organism was combined. Uninoculated controls were harvested similarly. Each filtrate was extracted 3 × (50 ml chloroform each extraction) and the three extracts combined and placed at 4 °C overnight. The chloroform layer was absorbed on a 35 g hydrophilic matrix column (Chem Tube CT-2050, Analytichem International, Harbor City, CA 90710) and gliotoxin was eluted.