Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Microbiological Methods 66 (2006) 286–293
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

A new and rapid bioassay for the detection of gliotoxin and related epipolythiodioxopiperazines produced by fungi

Olivier Grovel *, Isabelle Kerzaon, Karina Petit, Thibaut Robiou Du Pont, Yves-François Pouchus

S.M.A.B., Université de Nantes, Pôle Mer et Littoral- Faculté de Pharmacie, BP53508- 44035 Nantes cedex 01, France

Received 4 November 2005; received in revised form 6 December 2005; accepted 13 December 2005
Available online 31 January 2006

Abstract

Gliotoxin is an immunosuppressive cytotoxin produced by numerous environmental or pathogenic fungal species. For this reason, it is one of the mycotoxins which must be systematically searched for in samples for biological control.

In this study, a new, rapid and sensitive method for detecting gliotoxin has been developed. This bioassay is based on the induction of morphological changes in cultured cells (human KB cell line) by gliotoxin. Interpretation of the assay can be carried out after 1 h of incubation, either by direct microscopic observation, or with an automated microplate-reader at 630 nm. The limit of detection is 18–20 ng of gliotoxin in the well, depending on the used observation method. A high degree of specificity of the detection is brought about by the ability of the reducing reactant dithiothreitol to inhibit the biological activities of epipolythiodioxopiperazines (ETPs), such as gliotoxin, by reducing their polysulfide bridge. The bioassay allows a rapid primary screening of samples and a semi-quantitative evaluation of the gliotoxin concentration in extracts.

The method has been used to study the gliotoxin production by different fungal strains, allowing to highlight 3 strains of *Aspergillus fumigatus* producing gliotoxin in various extracts.

Keywords: Gliotoxin; Epipolythiodioxopiperazines; Bioassay; Fast detection; KB cell line; *Aspergillus fumigatus*; *Penicillium* sp.

1. Introduction

Gliotoxin (Fig. 1) is an epidithiodioxopiperazine (ETP) mycotoxin produced by a large number of fungi, such as *Trichoderma lignorum* (Weindling, 1932), *Trichoderma virens* (Anitha and Murugesan, 2005), *Penicillium obscurum* (Mull et al., 1945), *Gliocladium fimbriatum* (Johnson et al., 1943), *Candida albicans* (Shah and Larsen, 1991) and *Aspergillus fumigatus* (Glister and Williams, 1944). This compound is known to exhibit numerous biological activities, entirely dedicated to the oxidized form of the compound with an intact disulfide bridge. Indeed, structural studies have shown that the biological activities of gliotoxin – as most of the ETPs – involve the interaction of the polysulfide link with sulfur nucleophiles in a thiol-disulfide exchange (Waring and Beaver, 1996; Bertout et al., 2001). This bridge should then be in the oxidized form, the reduced dithiol form being inactive (Trown and Bilello, 1972; Yamada et al., 2000).

Gliotoxin was first described as an antibiotic because of its antimicrobial, antifungal and antiviral properties (Larin et al., 1965; Mc Dougall, 1969). It is lethal as from relatively low concentrations (Pahl et

* Corresponding author. Tel.: +33 2 40 41 28 51; fax: +33 2 40 41 28 58.
E-mail address: olivier.grovel@univ-nantes.fr (O. Grovel).

0167-7012/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2005.12.005

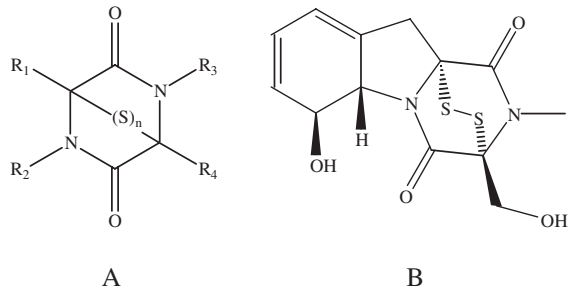

Fig. 1. Generic structure of epipolythiodioxopiperazines (ETPs) (A); structure of gliotoxin (B).

al., 1996) and is now recognized as being toxic for both humans and animals if accidentally ingested (Waring and Beaver, 1996). For instance, gliotoxin has been linked to a case of intoxication and death on camels having consumed contaminated hay (Gareis and Wernery, 1994). For this reason, it is recommended to detect its production in fungal suspensions used for biological control in the agricultural field.

Gliotoxin has been shown to inhibit some enzymes such as farnesyl-protein transferase, alcohol dehydrogenase and acetolactate synthase of higher plants and creatine kinase of rabbit muscle cell (Van Der Pyl et al., 1992; Waring et al., 1995; Haraguchi et al., 1996; Hurne et al., 2000). It has also been shown to inhibit the assembly and the function of the NADPH oxidase enzyme complex in human polymorphonuclear leukocytes (Tsunawaki et al., 2004; Nishida et al., 2005). It also exhibits an important immunosuppressive activity and is thought to play a role in the *A. fumigatus* virulence by facilitating fungal growth and colonization of host tissue through induction of a local or generalized immunosuppression (Pahl et al., 1996; Bondy and Pestka, 2000; Tomee and Kauffman, 2000; Daly and Kavanagh, 2002; Watanabe et al., 2003; Reeves et al., 2004). This toxicity against the immune system has been demonstrated in vitro on numerous cell lines: it induces apoptotic cell death of thymocytes, peripheral lymphocytes, macrophages, spleen cells and others, characterized for instance by DNA fragmentation and adduct formation (Waring et al., 1988; Waring, 1990; Sutton et al., 1994; Waring and Beaver, 1996; Golden et al., 1998; Lütjohann et al., 1998).

Its mode of action seems complex and is to this day still not clearly explained. It involves a glutathione-dependent uptake into cells and a redox cycling, resulting in modification of cellular Ca^{2+} influx and Mg^{2+} efflux from mitochondria (Hurne et al., 2002; Bernardo et al., 2003; Salvi et al., 2004). It was also demonstrated that gliotoxin inhibits the nuclear transcription factor NF-κB and the 20S proteasome activity, which might account for the immunosuppressive properties of this ETP (Pahl et al., 1996; Kroll et al., 1999).

In vitro, gliotoxin has been shown to inhibit cellular growth or to exhibit cytotoxicity with numerous cell lines (Waring et al., 1990; Richard et al., 1994; Shah et al., 1998; Zhou et al., 2000; Wenehed et al., 2003; Dewitte-Orr and Bols, 2005); but only some of those (L929, SK, MDCK and HeLa cells) have already been used for gliotoxin detection (Hanelt et al., 1994; Piva, 1994; Belkacemi et al., 1999). In these methods, the toxic response to the samples is evaluated by the remaining viability of the cells using a staining reaction after several hours of incubation (2–24 h). None of them offer a high specificity for gliotoxin detection and their sensitivity is low (0.4–1 μg/ml).

This paper describes the development of a new bioassay using the KB human epidermoid cell line, to improve rapidity, sensitivity and specificity for the detection of gliotoxin and related mycotoxins. This method has been applied to the study of gliotoxin production by different fungal strains on two kinds of extracts.

2. Materials and methods

2.1. Fungal strains

A marine *A. fumigatus* Fres. strain (M1) (No. LCP 99.4342, M.N.H.N. culture collection, Paris) was isolated from sediment from a mussel bed in the Loire estuary (France). As shown in a previous study, this strain produces gliotoxin in in vitro marine conditions (Grovel et al., 2002). Pathogenic *A. fumigatus* terrestrial strains (T1 and T2) were isolated from patients with bronchopulmonary disease in the Nantes University Hospital (France). Fifteen *Penicillium*sp. strains and eight *Aspergillus*sp. strains were isolated from marine sediments of Bourgneuf Bay (France) for a study of toxigenic fungi in shellfish farming areas (Sallenave-Namont et al., 2000).

2.2. Fungal cultures and preparation of samples

Fungi were cultured on solid medium (enzymatic digest of casein 10 g, dextrose 40 g, agar 15 g, natural seawater 1 l) (Becton Dickinson, Sparks, USA) and incubated at 27 °C for twelve days.

2.2.1. Solid medium extracts

Cultures were grown in 9-cm i.d. Petri dishes containing 25 ml of solid medium. Extracts were prepared according to the method described by Smedsgaard (1997). Three 6 mm i.d. plugs were recovered using a hollow punch and placed in 500 μl of a mixture of