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Antibacterial effect of the bioactive compound beauvericin produced by *Fusarium proliferatum* on solid medium of wheat

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ABSTRACT

To obtain the bioactive compound beauvericin (BEA), *Fusarium proliferatum* CECT 20569 was grown on a solid medium of wheat, utilizing the technique of the solid state fermentation (SSF), being this mycotoxin purified by high performance liquid chromatography (HPLC) with a reverse phase semi-preparative column using as the mobile phase acetonitrile/water in gradient condition. The purity of the BEA was verified by analytical HPLC and liquid chromatography tandem mass spectrometry (LC/MS-MS). The pure fractions of BEA were utilized to determinate the antibiotic effects on several bacterial strains that are considered normally pathogens of the intestinal tract as: *Escherichia coli, Enterococcus faecium, Salmonella enterica, Shigella dysenteriae, Listeria monocytogenes, Yersinia enterocolitica, Clostridium perfringens, Pseudomonas aeruginosa* and *Staphylococcus aureus.*
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1. Introduction

Mycotoxins are naturally occurring secondary metabolites produced by the mycelial structures of either toxigenic or nontoxigenic filamentous fungi (Paciolla et al., 2008). Beauvericin (BEA) is a depsipeptide that was first identified in a soil-borne entomopathogenic (insect-pathogenic) fungus *Beauveria bassiana*, which was recognized as the causative agent for heavy losses of the European sericulture in the 16th and 17th centuries (Tang et al., 2005). Nowadays, BEA is considered to be a putative mycotoxin (toxic fungal metabolite) that may affect human and animal health, since it is also produced by many species of the fungus *Fusarium* that infect important cereal grains such as corn, rice, and wheat (Desjardins et al., 2000; Logrieco et al., 1993a). The potential mycotoxic role of BEA is exemplified by results from *in vitro* studies using cell lines. For instance, BEA induces significant cell deaths in insect, murine, and human tumor cell lines (Mazziotti and Perlmutter, 1998; Calo et al., 2003). Furthermore, BEA is a potent and specific cholesterol acyltransferase inhibitor in rat liver microsomes (Tomoda et al., 1992). In mammalian cell lines, cell deaths caused by BEA have been suggested to involve a Ca^{2+} dependent pathway, in which BEA induces a significant increase in intracellular Ca^{2+} concentration that leads death cell as a result of a combination of both apoptosis and necrosis (Logrieco et al., 1998; Nilanonta et al., 2002; Jow et al., 2004; Lin et al., 2005; Ojcius et al., 1991).

The mechanism of BEA-induced Ca^{2+} increase, however, remains inconclusive. BEA-induced apoptotic changes such as DNA fragmentation have been demonstrated to take place in the complete absence of extracellular Ca^{2+} (Ojcius et al., 1991), suggesting that BEA triggers release of Ca^{2+} from internal Ca^{2+} stores. In fact, BEA has since been regarded as an apoptotic agent that releases Ca^{2+} exclusively from endoplasmic reticulum (Lin et al., 2005).

BEA has exhibited minimum inhibitory concentration (MIC) values of 0.8–1.6 μg/mL against *Mycobacterium tuberculosis*, and IC_{50} values of 1.3–2.4 μg/mL against *Plasmodium falciparum* (Nilanonta et al., 2002). In a recent

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study, the combined use of BEA with ketoconazole (an antifungal drug) synergized or enhanced the antifungal effect, suggesting the potential use of BEA as a co-drug for antifungal infections in human (Zhang et al., 2007). A gene responsible for BEA biosynthesis has been recently isolated from a *B. bassiana* strain and confirmed through heterologous expression of the gene for BEA synthesis in *Escherichia coli*; the BEA biosynthesis has been shown to play a very significant role for the insecticidal activities of the fungus (Xu et al., 2008).

As regard the antibacterial activity of the BEA, Castlebury et al. (1999) studied the action of this bioactive compound on bacteria typically isolated from mammalian intestinal tract to understand the interaction of the BEA with the normal intestinal microbiota that has an important role in nutrition, physiology, and colonization resistance to invasive pathogens. Also, Fotso and Smith (2003) evaluated the BEA mutagenicity, utilizing the Ames test on *Salmonella typhimurium* standard tester strain.

In particular, considering the few works that are published on the interaction of the BEA and bacteria, the aim of this study was: (i) to purify and characterize the BEA produced by *Fusarium proliferatum* on solid culture of corn and (ii) to determinate the antibiotic effects of the BEA on several bacterial strains.

2. Materials and methods

2.1. Chemicals

Acetonitrile, methanol, hexane, chloroform, and 2-propanol were purchased from Merck (Whitehouse Station, N.J., U.S.A). Deionised water (<8 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. Potato dextrose broth (PDB) was obtained from Oxoid (Basingstoke, UK). BEA was provided from Sigma Aldrich (Madrid, Spain).

2.2. Fusarium *strain and culture conditions*

The strain of *F. proliferatum* CECT 20569 is obtained at the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol. A solid medium of wheat was utilized in this study. Briefly, one hundred grams of solid wheat was weighted in a 2 L Erlenmeyer flasks, and adding 2 L of water. The suspensions were boiled for 15 min, and filtered with paper filter Phenomenex (Torrance, Calif.) to separate the liquid fraction from the solid (De la Luz et al., 2007). The solid fraction was autoclaved for 20 min at 121 °C, and a suspension of 10^6 conidia mL^{-1} of *F. proliferatum* CECT 20569 growth during three days in a PDB preinoculum, was used for inoculation of the medium. Conidial concentration was measured by optical density at 600 nm and adjusted to 10^6 conidia mL^{-1} in the Erlenmeyer flasks (Kelly et al., 2006). The fermentations were conducted at the temperature of 25 °C on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany) in batch culture for 30 days. At the end of the fermentations the solid culture was extracted for the analysis of the cyclohexadepsipeptides.

2.3. Sample preparation

Sample preparation was performed as described by Jestoi et al. (2007). Fermented wheat (50 g) with 200 mL of acetonitrile was extracted with the Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. Then, samples were centrifuged at 4000 rpm for 5 min. Five milliliters of crude extract was cleaned with SPE using a silica column. Before loading the sample onto the column, which was conditioned with acetonitrile (2 mL). A sample was passed through the conditioned column and the eluate was collected into a test tube. The column was further washed with acetonitrile (2 mL) being also collected. The combined eluates were concentrated under the steam of nitrogen, dissolved into methanol and injected in the liquid chromatographic equipment.

2.4. Purification of BEA by semi-preparative column LC

The BEA extract was dissolved in methanol and the purification with semi-preparative LC were performed using a LC system equipped with LC-7100 pump, autosampler L-2200 (200 μL loop) and a diode-array detector (DAD) L-7455 from Hitachi (Tokyo, Japan). A Gemini C18 column (250 × 10 mm, 5 μm) (Phenomenex, Torrance, CA) was used and a mobile phase with acetonitrile-water gradient at flow rate of 3.0 mL/min. Solvent program was started with 70% acetonitrile, held for 5 min, followed by a linear gradient to 90% acetonitrile in 10 min. BEA was detected at 205 nm. The end of the column was connected at a fraction collector FC 204 from Gilson (Middleton, USA). Liquid chromatographic retention time and UV absorbance spectrum of the purified BEA was compared to those of the crude sample (97%) provided by Sigma Aldrich (Madrid, Spain). The LC-purified BEA solution was concentrated on a rotary evaporator at 35 °C. The concentrated BEA solutions was frozen and stored at −20 °C.

2.5. LC evaluation of the BEA collected

The amount of BEA recovered was evaluated with the previous chromatographic equipment and with an analytical column, Gemini C18 column (250 × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA). The mobile phase was acetonitrile/water (80:20 v/v), at the flow rate of 1 mL/min in isocratic condition. The UV diode-array detector was set at 205 nm (Monti et al., 2000). Samples were dissolved in methanol.

2.6. LC–MS/MS confirmation analysis of produced ENs

LC analysis of BEA was carried out with a TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface. Mass Lynx NT software 4.1 was used for data acquisition and processing. The autoinjector was

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