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Purification of an antifungal compound, cyclo(L-Pro-D-Leu) for cereals produced by *Bacillus cereus* subsp. *thuringiensis* associated with entomopathogenic nematode

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ABSTRACT

Mold spoilage is the main cause of substantial economic loss in cereals and might also cause public health problems due to the production of mycotoxins. The aim of this study was to separate and purify and to identify antifungal compounds of bacterium associated with novel entomopathogenic nematode and check the antifungal property of identified compound in particular food model systems. The antifungal compound was purified using silica gel column chromatography, TLC and HPLC and its structure was elucidated using NMR (^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMBC), HRMS and Marfey's method. Based on the spectral data, the active compounds were identified as diketopiperazine [cyclo(L-Pro-D-Leu)]. The antifungal activity of cyclo(L-Pro-D-Leu) was studied by MIC and paper disk assay against *Aspergillus flavus* MTCC 277 and *Aspergillus niger* MTCC 282 and best MIC value of 8 μg/ml was recorded against *A. flavus*. Cyclo(L-Pro-D-Leu) strongly inhibit mycelia growth of fungus and thereby affecting aflatoxin production. To investigate the potential application of the cyclo(L-Pro-D-Leu) and to eliminate fungal spoilage in food and feed, soybean and peanut were used as models. White mycelia and dark/pale green spores of *A. flavus* were observed in the control soybeans after 2-day incubation. However the fungal growth was not observed in soybeans treated with cyclo(L-Pro-D-Leu). Almost the same result was observed for peanuts treated with cyclo(L-Pro-D-Leu) for *A. niger*. The cyclo(L-Pro-D-Leu) was nontoxic to two normal human cell lines (FS normal fibroblast and L231 lung epithelial) up to 200 μg/ml. Thus the diketopiperazine derivative identified in the study may be a promising alternative to chemical preservatives as a potential biopreservative which prevent fungal growth and mycotoxin formation in food and feed.

Introduction

Molds and yeasts are important spoilage organisms in food products and feed systems. Up to 5–10% of the world's food production is lost because of fungal spoilage (Pitt and Hocking 1999). *Aspergillus* species are one of such mold which is responsible for several disorders in various plant and plant products. The most common species that cause food spoilage are *Aspergillus flavus* and *Aspergillus niger*, followed by *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus carbonarius*, and *Aspergillus alliaceus*. They can contaminate agricultural products at different stages including pre-harvest, harvest, processing and handling (Perrone et al. 2007).

A. flavus and *A. niger* are soil-inhabiting, filamentous fungus that saprophytically utilizes a wide range of organic substrates. Though *A. flavus* is considered as a saprophyte, it is also well known as an opportunistic pathogen in humans (Mellon et al. 2007). *Aspergillus* sp. can invade important oil seed crops such as soybean, peanut, corn, and cotton seed. They are also encountered as storage molds on plant products (Kozakiewicz 1989). Apart from this, *Aspergillus* species secrete several toxic secondary metabolites in foods. The toxic secondary metabolites produced by *Aspergillus* species contaminated in foods and feeds are the aflatoxins, the most potent natural carcinogen known (Squire 1981; Trail et al. 1995; Payne and Brown 1998; Sweeney and Dobson 1999) and ochratoxin A (Varga et al. 1993). Aflatoxins B1, B2, G1, G2 are the toxic naturally occurring mycotoxins secreted by *A. flavus*. Due to their extreme hepatocarcinogenicity, aflatoxins pose a risk to human health because of their extensive pre-harvest contamination of soybean, peanuts, corn, cotton, and tree nuts. Aflatoxin residues from contaminated feed may even appear in milk. The other changes due to the spoilage of *Aspergillus* sp. can be of sensorial, nutritional and qualitative nature like pigmentation, discoloration, rotting, development of off-odors and off-flavors (Perrone et al. 2007).

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Xenorhabdus and *Photorhabdus*, members of the family Enterobacteriaceae, engage in a mutualistic association with the entomopathogenic nematodes (EPN) *Steinernema* and *Heterorhabditis*, respectively, and are also pathogenic toward different insect hosts (Goodrich-Blair and Clarke 2007; Herbert and Goodrich-Blair 2007). The mutualistic bacteria, which are carried in the intestinal tracts of the nematode vector, are transported into the hemocoel of the insect host, which then usually dies within 48 h (Herbert and Goodrich-Blair 2007). The main causes of the host insect death are thought to be the combined actions of bacterial multiplication, septicemia, and toxins secreted by mutualistic bacteria (Sicard et al. 2004). The bacteria multiply rapidly and produce various metabolites that can overcome the insect immune system (Forst and Nealson 1996), kill the insect, and inhibit the growth of various fungal and bacterial competitors (Chen et al. 1996). By doing so, the bacterial symbionts are believed to prevent putrefaction of the insect cadaver and establish conditions that favor the development of both the nematode and bacterial symbionts (Gaugler and Kaya 1990).

Various kinds of antibiotics against bacteria and fungi are synthesized and secreted by *Xenorhabdus* and *Photorhabdus* (Webster et al. 2002). Most species of *Xenorhabdus* and *Photorhabdus* produce more than one group of bioactive secondary metabolites and the metabolites from *Xenorhabdus* species are more diverse from *Photorhabdus*. It became obvious that these bacteria are in fact very productive secondary metabolite producers, which can produce several structurally diverse compounds. These metabolites not only have diverse chemical structures, but also a wide range of bioactivities of medical and agricultural applications (Wang et al. 2011). Therefore entomopathogenic bacteria can be regarded as a novel source of potential pharmaceuticals which have been studied only recently. In the course of studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Oscheius* was isolated from sweet potato weevil grubs collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram. A specific bacterium was found associated with the nematodes. The nematodes could be cultured on laboratory reared *Galleria mellonella* larvae and maintained alive for several years. The bacteria were found to be pathogenic to a number of insect pests (Mohandas et al. 2007). The cell free culture filtrate of the bacteria was found to inhibit several pathogenic bacteria, fungi and a plant parasitic nematode (*Meloidogyne incognita*) (Mohandas et al. 2007), suggesting that it could be a rich source of biologically active compounds.

The aim of this study was to purify and to identify the antifungal compound produced by *Bacillus* sp. associated with novel entomopathogenic nematode, and to determine if the compounds produced by the bacterium have the capacity to prevent fungal growth in a soybean and peanut food model system.

Materials and methods

Chemicals and media

All the chemicals used for extraction and column chromatography were of analytical grade and High performance liquid chromatography (HPLC) grade methanol was from Merck Limited, Mumbai, India. Microbiological media were from Hi-Media Laboratories Limited, Mumbai, India. All other reagents were of analytical grade and the other chemicals used in this study were of highest purity. The software used for the chemical structure drawing was Chemsketch Ultra, Toranto, Canada.

Test fungus

A. flavus MTCC 277 and *A. niger* MTCC 282 were purchased from Microbial Type Culture collection Centre, IMTECH, Chandigarh, India and were grown on potato dextrose agar (PDA) at 30 °C for 3 days and stored at 4 °C.

Antifungal producing bacteria

The antifungal compound-producing bacteria were isolated from 3rd stage infective juveniles of the nematode sample collected from sweet potato weevil grubs or from the hemolymph of nematode infested *G. mellonella* larvae. The strain was identified as *Bacillus cereus* subsp. *thurigenesis* (Accession No. CP001407) based on 16S rDNA and BLAST analysis. The strain was currently deposited in IMTECH (Institute of Microbial Technology, Chandigarh; India) and the accession number is MTCC 5234.

Preparation of cell-free supernatant

The bacterial fermentation was carried out using modified tryptic soya broth (TSB) (tryptone 17 g/l, soytone 3.0 g/l, glucose 2.5 g/l, NaCl 5.0 g/l, meat peptone 10 g/l, water 1000 ml). A single colony of *Bacillus* sp. N strain from the nutrient agar plate was inoculated into the flask containing 100 ml sterile media. The flasks were incubated in a gyrorotatory shaker (150 rpm) at 30 °C in dark for 24 h. When the optical density of the culture at 600 nm was approx 1.7, the bacterial cultures were transferred aseptically into 400 ml sterile medium and incubated in the gyrorotatory shaker at 30 °C in dark for 96 h. The culture media were then centrifuged (10,000 × *g*, 20 min, 4 °C) followed by filtration through a 0.45 μM filter, to obtain cell free culture filtrate. Thirty liters of cell free culture filtrate were neutralized with concentrated hydrochloric acid and extracted with an equal volume of ethyl acetate thrice. The ethyl acetate layers were combined, dried over anhydrous sodium sulfate, and concentrated at 30 °C using a rotary flash evaporator. The initial antifungal activity of the crude extract was tested against *A. flavus* by paper disk diffusion method.

Purification of antifungal compounds

The oily yellow residue (9.3 g) obtained after drying was then loaded on a silica gel column (25 mm × 600 mm) previously equilibrated with hexane and eluted successively with 200 ml of 100% hexane, 200 ml of linear gradient hexane: dichloromethane (v/v, 75:25 to 25:75), 200 ml of 100% dichloromethane, 200 ml of linear gradient dichloromethane:ethyl acetate (v/v, 95:5 to 5:95), 200 ml of 100% ethyl acetate and finally with 200 ml of 100% methanol. Two fractions (100 ml each) were collected from each combination. Purity of the compounds were tested by thin layer chromatography (20–20 cm, precoated silica gel 60 GF_{254} plates, Merck Limited, Germany) and high pressure liquid chromatography using LC-10AT liquid chromatograph (LC; Shimadzu, Singapore) equipped with a C-18 column (Shimadzu, Singapore, 5 μm, 4.6 mm × 250 mm, 1.0 ml/min) and 100% methanol as a mobile phase.

Structure determination of antifungal compound

UV spectrophotometer

UV–vis spectrum of the pure compounds was recorded on a Systronics double beam spectrophotometer 2201, India at room temperature (scanning range 190–800 nm).

Nuclear magnetic resonance (NMR)

The structure of the compounds was determined using nuclear magnetic resonance (NMR) spectroscopy (Bruker DRX 500 NMR instrument, Bruker, Rheinstetten, Germany) equipped with a 2.5-mm microprobe. NMR Spectrometer using $CDCl_3$ was deployed to measure ^1H and ^{13}C and 2D NMR. All spectra were recorded at 23 °C. One-dimensional ^1H NMR experiments as well as two-dimensional