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Production and characterization of Destruxins from local *Metarhizium anisopliae var. anisopliae*

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Abstract: Characterization of insecticidal cyclodepsipeptides, destruxins A and B, from *Metarhizium anisopliae* fermentation broth was performed. The isolation scheme of these destruxins using ion-exchange chromatography, silica gel chromatography is present. The quality of semi-preparative products was unique; over 90% purity was achieved for broth destruxins A and destruxins B. Purified destruxins were further identified employing the mass spectrophotometer. The used of the purified destruxins as analytical standards demonstrated good correlation.

Key words: *Metarhizium anisopliae*; Destruxins; Mycotoxins; Toxins; Chromatography

INTRODUCTION

Fungi belonging to the genera *Metarhizium* have been found to be effective against several species of insects, including Lepidoptera (Loutelier *et al*. 1996). Destruxins are a class of hexadepsipeptides produced by different soil fungi including *Metarhizium anisopliae*, and most of the more than 35 characterized congeners have been described from this species (Pedras *et al*. 2002). Destruxins can be classified into main series, depending on the nature of the pentanoic acid side chain and into further sub-series according to differences in the amino acid substitution pattern. Among these, Destruxins A, B, and E are predominating in quantitative terms, which have the same amino acid sequence but differ in their hydroxyacid residue. In addition to the insecticidal activity, the most important congener Destruxins E has been reported to prevent the formation of osteoblasts (Nakagawa *et al*. 2003), inhibit the proliferation of certain cancer cell lines (Morel *et al*. 1983; Odier *et al*. 1992; Kobayashi *et al*. 2004), and intervene during the disease by an immune-inhibitory effect (Vey *et al*. 2002). The mode of action is still under investigation, but Destruxins E is known to inhibit vacuolar-type H (+)-ATPase activity (Togashi *et al*. 1997; Yoshimoto and Imoto 2002). Furthermore, dtx E has also been reported to influence calcium fluxes and protein phosphorylation (Dumas *et al*. 1996), insecticidal and cytotoxic (Dumas *et al*. 1994).The fungus *Metarhizium anisopliae* produces a family of cyclic peptide toxins termed destruxins, which exhibit various insecticidal activities to a wide range of insects. This entomopathogenic fungus is found in soils throughout the world. It was first recognized as biocontrol agent in the 1880s. *M. anisopliae* is a major source for producing the destruxins Gupta *et al.* (1989) in addition to agricultural use, some investigators have found that destruxins may induce erythropoietin production Cai *et al.*(1998), antiviral effects Calms *et al.* (1993) & Chen *et al.* (1995). Dtxs represent a class of cyclic depsipeptides that potentially can be used for varieties of applications, such as insecticidal, cytotoxic, antiviral, immunosuppressant and phytotoxic activities. By virtue of their structure, dtxs exhibit a wide spectrum of biological and insecticidal activities (Pedras *et al*., 2002; Sowjanya *et al*., 2008b). Dtxs also cause signal transduction changes through the phosphorylation of certain proteins in lepidopteran and human cell lines (Dumas *et al*., 1996). Upon dtx treatment, the remarkable changes in the activity of antioxidant enzymes and lipoxygenase as well as the levels of lipid peroxidation were found in *S. litura* larvae, implied that the exposure of larvae to dtx could induces oxidative stress (Sowjanya Sree and Padmaja 2008). In this work, the isolation and purification of destruxins A and B from local *Metarhizium anisopliae* var. *anisopliae*

MATERIALS AND METHODS

Microorganism and Cultivation:

Metarhizium anisopliae var. *anisopliae* was isolated from red palm weevil, *Rhynchophorus ferruginens* in Ismailia governorate, Egypt (Ibrahim, 2006). The spore suspension used as inocula was obtained from 5 day-old submerged cultures grown on 3.5% Czapek-Dox broth (Difco, Detroit, MI, USA) supplemented with 0.5% peptone, at 200 rpm and 28˚C. For submerged fermentation, 5% (v/v) inocula level was used. The cultivation was performed with a 500mL Erlenmeyer flask containing 100 mL of 3% maltose and 0.5% peptone liquid medium and the culture was allowed to grow at ambient temperature (28˚C) for 14 days on a rotary shaker (150 rpm).

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Isolation and Purification of Destruxins:

The mycelium was separated by filtration through cotton cloth. The supernatant was then extracted three times with equal volume of methylene dichloride. The organic layer was collected, and concentrated with a vacuum rotary evaporator. This crude extract was then passed through an Amberlite cation-exchange chromatographic column into another Amberlite IR 120 Plus anion exchange column without further treatment. This column was also washed with distilled water under gravity mode. Pooled eluted sample was re-dissolved in an equal volume of acetonitrile, and concentrated using a vacuum rotary evaporator. Acetonitrile-soluble residue was flash chromatographed on a Merck K-60 silica gel column (230–400 mesh) employing a 40 mL step-wise chloroform –methanol (99: 1.0) solvent gradient to give desired polarities. All solvents used were degassed and filtered through a 0.22 mm filter before use. Fractionated samples were characterized using Spectrophotometer UV-visible 2401PC (Shimadzu, Japan).

Mass Spectrophotometer:
Specifications:

The HPLC-ESI-MS system consisted of an HP 1100 Series HPLC instrument (quaternary pump and degasser, column compartment, and autosampler) and an LCQ ADVANTAGE MAX mass spectrometer from Thermo Finnigan "Xcalibure 1.4" software.

Tuning Method:

Source voltage; 4.5 KV, source current; 100 uA, capillary voltage; 3 V, capillary temperature; 200 ºC, sheath gas flow; 60 arb., Helium was used as a collision gas at a pressure of 2.5 to 3.5 mTorr, the collision energy was set to 25 eV, tube lens offset; 5 V, octapole RF amplifier 500 Vp-p, octapole offset (1); -2.5 V, octapole offset (2); 7 V, entrance lens; -20 V, and interoctapole lens voltage; -40 V. Full scan for the ionized sample was applied and MS/MS fragmentation for the mostly abundant peaks using collision gas (Helium) and at a collision energy.

RESULTS AND DISCUSSION

Destruxins are synthesized by different species of entomopathogenic fungi and are potent toxins (Amiri *et al*., 2000) with bioactivity against various insects. The destruxins can be administered by application to the external surface of the cuticle, ingestion, injection, or any combination of these routes, to *S. litura* larvae (Sowjanya *et al*., 2008a) or *B. tabaci* nymphs (Hu *et al*., 2009). Early work has shown that destruxin A has strong contact toxicity on *E. serrulata* (Hu *et al*., 2007), and destruxin E can induce the formation of vesicles on microvilli of the apical cell surface, pycnosis of the nucleus and changes of mitochondrial shape and density (Dumas *et al*.,1996a).

The medium used in the submerged culture studies of *Metarhizium anisopliae* var. *anisopliae* has been focused on the combination of Czapek-Dox broth and peptone with no exception in the literature. A modified medium was used to grow this fungus. *Metarhizium anisopliae* var. *anisopliae* was cultured on a maltose medium containing 0.5% peptone in a flask shake at 150 rpm. The maltose used as a main carbon source gave a higher production of destruxin. Mycelium from the 14-day old culture of *M. anisopliae* var. *anisopliae* was separated by filtration through cotton cloth. The supernatant was extracted three times by equal volume of Chloroform. This crude extract was then passed through an Amberlite cation-exchange chromatographic column into another Amberlite IR 120 Plus anion exchange column chromatography.

The sample was separated by two-step ion-exchange chromatography. Combination of anionic and cationic exchange provided another step to removing charged impurities existed in the mixture. The crude extract collected from ion-exchange column was subjected to flash silica gel chromatography as a pre-purification step. The column was eluted with a mixture of chloroform–methanol (99:1.0), and then with increasing proportion of methanol in the mixture until 5%. **Figure (1)** shows fractionated silica gel chromatographic plots for the five concentrations of chloroform. Most of peaks were washed out at the chloroform level of 98%... In practice, two major Destruxins (A & B) were isolated from the first silica gel fraction. As evident from the chromatogram these destruxins showed excellent resolution on cation-exchange under the experimental conditions. Further purification was done by loading the sample from silica gel column chromatography. The elution delivered from a single peak were collected and concentrated.

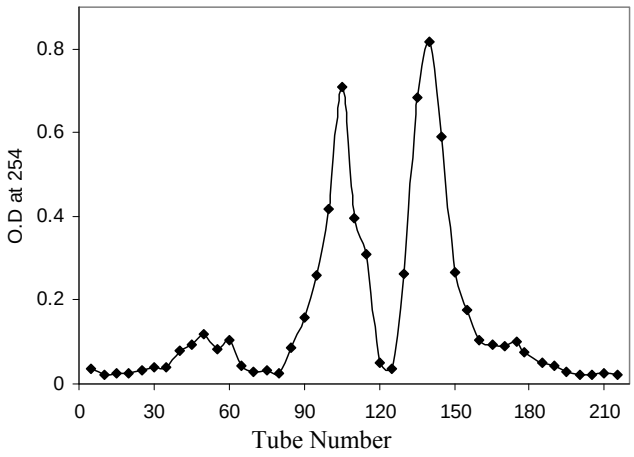

Fig. 1: Typical elution profile for the chromatography of destruxin A and B on Cation-exchange chromatographic column into another Amberlite IR 120

Mass Spectrophotometer:

The MS spectrum of purified fractions of the soluble destruxins A and B produced from cultivation of *Metarhizium anisopliae* var. *anisopliae*. Purified fraction from the culture gave a peak of m/z 578 corresponding to A (**Figure 2**). Moreover, a very strong peak appearing in the position of m/z 594 corresponds to B **(Figure 3).** Although E has a similar molecular mass, its structure is quite different in the substitution group (Gupta *et al*., 1989 and Loutelier *et al*., 1996). Comparison of the retention time on HPLC also suggested that no E peak was obtained. Similarly, A shows protonated molecules at m/z 577, which is similar to the data reported elsewhere [16]. Thus, the results of the mass spectral analysis,

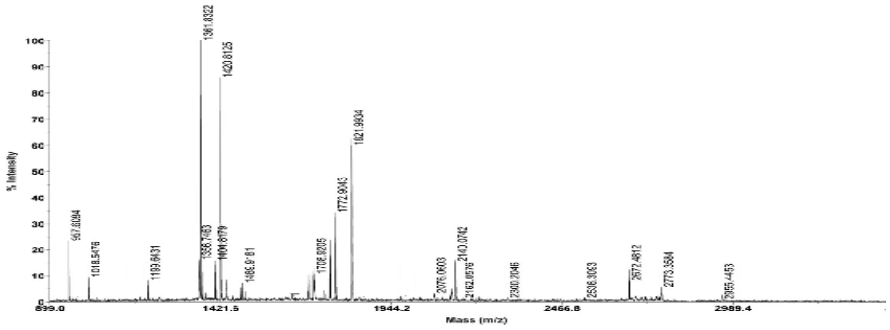

Fig. 2: MS spectrum of purified fractions of the destruxins A

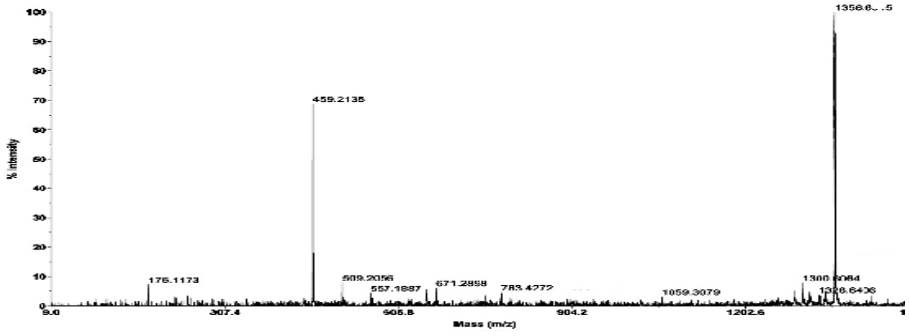

Fig. 3: MS spectrum of purified fractions of the destruxins B.

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