SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

A Sensitive Bioassay for Destruxins, Cyclodepsipeptides from the Culture Filtrates of the Entomopathogenic Fungus *Metarhizium anisopliae* (Metsch.) Sorok.

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Bioensaio Sensível para Destruxinas, Ciclodepsipeptídes do Filtrado de Culturas do Fungo Entomopatogênico *Metarhizium anisopliae* (Metsch.) Sorok.

RESUMO - Destruxina A, um ciclodepsipeptídeo tóxico, foi purificado a partir de filtrados de culturas do fungo entomopatogênico *Metarhizium anisopliae* (Metsch.) Sorok. pelo método de HPLC, e seus efeitos foram testados em uma preparação de coração da lagarta *Manduca sexta* (L.) (Lepidoptera: Sphingidae). Destruxina A causou uma aceleração da pulsação de forma reversível e dependente da dose. O bioensaio com o coração foi muito sensível a aplicações pulsáteis de destruxinas mesmo em concentrações muito baixas, como 35 pM. Devido à sua elevada sensibilidade, o bioensaio com o coração de *M. sexta* pode ser usado para detectar e quantificar estas toxinas em extratos de tecidos de insetos infectados pelo fungo ou para confirmar a atividade biológica de frações de HPLC. O sítio de ação da Destruxina A no coração não foi determinado. Porém, foi confirmado que esta toxina não teve ação no receptor 5HT.

PALAVRAS-CHAVE: Insecta, *Manduca sexta*, coração, toxina, peptídeo, destruxina.

ABSTRACT - Destruxin A, a toxic cyclodepsipeptide, was purified from culture filtrates of the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorok. by Reverse-Phase HPLC, and assayed for its effects on a larval *Manduca sexta* (L.) (Lepidoptera: Sphingidae) heart preparation. Destruxin A was found to cause reversible and dose-dependent acceleration of the heart beat. The heart bioassay was highly sensitive to pulse applications of destruxin A at concentrations as low as 35 pmol. Because of its high level of sensitivity, the *M. sexta* heart bioassay could be used to detect and quantify destruxins in tissue extracts from mycosed insects or to confirm bioactivity of HPLC fractions. The site of action of Destruxin A was not determined. However, this toxin did not act as a 5HT receptor agonist.

KEY WORDS: Insecta, *Manduca sexta*, heart, toxin, peptide, destruxin.

Destruxins (DTXs), a group of cyclic depsipeptides produced by *Metarhizium anisopliae* (Metsch.) Sorok., are the most well studied of the insecticidal compounds produced by entomopathogenic fungi. This is probably due to the potential of *M. anisopliae* as a biological control agent and the ease of culture of this fungus *in vitro*. Currently, DTXs are the only toxic compounds which have been proved to be present in mycosed insects in sufficient quantities to have caused host death (Suzuki *et al.* 1971; Samuels *et al.* 1988a). Destruxins are neither antibiotic (Tamura *et al.* 1964), nor do they act as ionophores (Samuels *et al.* 1988b), but are thought to act as neuromuscular toxins. Destruxins also have an action on insect defence mechanisms, inhibiting the phagocytic activity of plasmatocytes of *Galleria mellonella* (L.) (Vilcinskas *et al.* 1997).

Variations among methods of separation or use of different isolates of *M. anisopliae* have resulted in differences in the types and quantities of DTXs isolated. For example Pais *et al.* (1981), using fermenter scale cultures of *M. anisopliae*, identified 14 DTXs from their mass spectrometry profiles. Bioassay of these DTXs was not performed probably due to the lack of an adequate method for detecting and quantifying activity. One of the standard bioassays for insecticidal activity of DTXs has been the use of knockdown ED_{50} type testing (Samuels *et al.* 1988a). This type of bioassay is labour intensive, time consuming, and dependent on the insect species, and can only be used to assay for DTX in microgram quantities. We have found that from a range of insect species tested, only lepidopteran larvae and dipteran adults were sensitive to intrahaemocoelic injection of DTXs at concentrations in the range of 10 mg g^{-1} insect (Samuels *et al.* 1988a). A new technique for toxicity testing of compounds by ingestion demonstrated that low concentrations of DTXs (18 mg ml^{-1}) were toxic to the silverleaf whitefly *Bemisia argentifolii* (Bellows & Perring) by ingestion (Davidson *et al.* 1996). However, this technique could not be used for quantifying either extremely low concentrations of DTXs isolated from infected/moribund insects or the bioactivity of some of the DTXs produced in low quantities in culture filtrates. Therefore, a new highly sensitive bioassay was developed using *Manduca sexta* (L.) (Lepidoptera: Sphingidae) heart preparation, which gave a rapid and quantifiable response to applications of DTX.

Materials and Methods

Isolation and Purification of Destruxins. The method used to obtain DTX from *M. anisopliae* culture filtrates (CF) was modified from that described by Roberts (1969). *M. anisopliae* isolate ME1 (host, *Curculio caryae* Horn, Coleoptera), supplied by Tate and Lyle Ltd., UK, was cultured on Czapek-Dox liquid modified medium (Oxoid) to which 0.5% Bacto-peptone (Oxoid) was added. Following 4 days of incubation at 27°C and shaking at 150 rpm, the DTXs were extracted from the CF using carbon tetrachloride (5x at 10:1; CF:CCl$_4$). The solvent was then removed using a rotary evaporator, leaving a residue that contained DTX and impurities.

The crude extract was passed through a column containing the cation exchange resin Dowex 50 W-X8 20-50 mesh (hydrogen ion form) at a rate of 1 ml min^{-1}. UV absorption (206 nm) was used to detect compounds not retained on the resin. The DTX fraction was determined by TLC by comparison to pure standards. The DTX fraction was then passed through an anion exchange resin (Bio-Rad Ag 1-X8 20-50 mesh, acetate form) and developed with deionized water at a flow rate of 1 ml min^{-1}. Fractions with absorbance in the 206 nm range were freeze-dried, redissolved in acetone, and approximately 1 ml (5mg ml^{-1} destruxins) run on TLC (Merck Keiselgel 60, F254, 10 x 20 cm; 19:1 chloroform:methanol) for 2h. DTX was visualized with iodine vapor, scrapped off the plate and suspended in deionized water, vortex mixed, and centrifuged at 3000 xg for 3 min. The resulting supernatant was freeze-dried in preparation for HPLC.

HPLC apparatus consisted of a Gilson system with two model 302 pumps and a Rheodyne injection valve. Detection was performed by UV using a Gilson Holochrome Model HM at 210 nm. The detector output was recorded using a flat bed chart recorder. For semi-preparative HPLC the support material was Spherisorb C_{18} 5mm (HPLC Technology) prepacked in a 30 cm x 8 mm i.d. column. For analytical work the same support material was used but packed into a 25 cm x 4.6 mm i.d. column. The HPLC was run in isocratic mode with 50% acetonitrile (Koch Light) and 50% Milli-Q water at 1 ml min^{-1} for analytical separations and 3.25 ml min^{-1} for semi-preparative isolations. The DTX A peak was collected manually and solvents removed by freeze drying. Concentration of DTX was calculated by comparison of peak areas with that of known concentrations of pure standards. Preliminary experiments were also carried out using pure DTX B, purified at the same time as DTX A, although its effects on *Manduca* heart are not shown here.

Caterpillar Heart Preparation. The *Manduca* heart preparation was originally developed by Platt and Reynolds (1985) for investigation of the mode of action of cardioactive peptides and was adapted here for bioassay of DTXs. Fifth instar larvae weighing between 8 and 10 g were taken from the colony of *M. sexta* which has been maintained on artificial diet (Bell and Joachim 1976) and kept under a long day (L:D, 17:7) photoperiod. The insects were anaesthetized by submersion in water for 15 min. The body wall was cut laterally, ventral to the spiracles so that the entire dorsal surface could be removed and pinned out in a Sylgard (Dow-Corning) dish. The gut was removed and the preparation was washed extensively with several changes of *Manduca* saline (in mmol^{-1}) (KCl, 40; NaCl, 4; MgCl$_2$, 18; CaCl$_2$, 3; Na$_2$HPO$_4$, 1.5; NaH$_2$PO$_4$, 1.5; sucrose, 193; pH 6.5)

Movement of the semi-isolated dorsal vessel was monitored by the deflection of a small hook (made from a 1A entomological pin) which was inserted underneath the heart between abdominal segments 5 and 6. This was connected by a light cotton thread to the lever of a Palmer isotonic movement transducer, lightly counterweighted (160 mg) with modelling clay. The transducer's output was registered on a flat bed chart recorder. The preparation was held at an angle of approximately 25^0 to the horizontal and fresh *Manduca* saline perfused continuously onto the caudal end of the heart at a rate of about 10 ml min^{-1}. The saline flowed down the length of the heart under gravity and was removed by suction at the cephalic end. All test substances were dissolved in *Manduca* saline prior to use. Serial dilutions of pure DTX A (20-500 ng), 10^{-6} M 5HT (Sigma, UK) and 1 unit of Central Nervous System (CNS) Extract (concentration not determined; for details see Platt and Reynolds, 1985), were applied directly to the preparation at the caudal extremity of the heart in a 50 μl pulse, using a 100 μl Hamilton microsyringe. 10^{-4} M 2-bromolysergic acid diethylamide (BOL) (Sigma, UK) was perfused over the heart preparation for 2 h before application of test extracts. The dose response curve was calculated from 4 concentrations of DTX A, replicated 3 times, using the same heart preparation. All assays were performed at room temperature (22$\pm$2^{0}C).

Results and Discussion

From the chromatogram (Fig. 1) demonstrating the separation of DTXs by Reverse Phase-HPLC, it can be seen that 4 DTXs were detected, however DTX A accounted for approximately 80% of the mixture. The semi-isolated *Manduca* heart preparation displayed regular, rhythmical contractions. Typically, there were 12-18 beats min^{-1}, which was approximately half the frequency measured by direct observation or by electrocardiography in the intact animals (Platt and Reynolds 1985).

DTX A increased the frequency and amplitude of *Manduca* larval heart beat (Fig. 2 A.I). When high concentrations (500 ng) were applied, the heart became paralysed tempo-
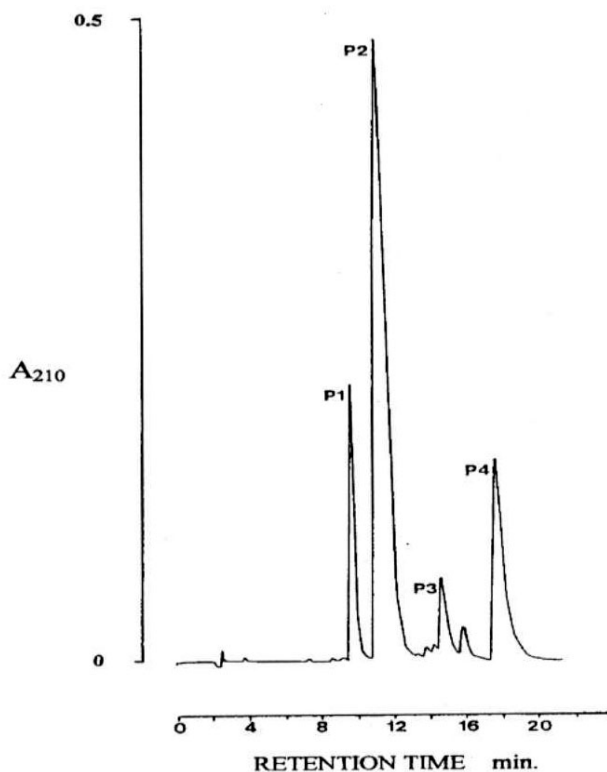
Figure 1. HPLC chromatogram demonstrating the separation of four destruxins. P1=Destruxin A$_2$; P2=Destruxin A; P3=Desmethyl-destruxin B; P4=Destruxin B.

rarily in systole and the baseline recovered only after prolonged periods of perfusion with *Manduca* saline (Fig. 2 A.III); therefore, these high doses were avoided. The heart preparation was found to be extremely sensitive to DTX, causing accelerated heart beat when exposed to pulse applications of only 20 ng (ca. 35 pmol) in a volume of 50 µl (Fig. 3). Therefore, this preparation could be used in the bioassay of small quantities of DTX present in extracts from mycosed insects or for confirmation of the activity of DTXs separated by HPLC. In a preliminary experiment to compare the cardioactive effects of other DTXs, DTX B was also found to cause accelerated heart beat at similar concentrations

to DTX A (results not shown).

The frequency of *Manduca* heart beat increased above the resting rate with increasing concentrations of DTX A, in the range 20 ng - 100 ng, with a typical dose response curve (Fig. 3). The effects of DTX A on the heart beat were compared to the effects of 5HT and cardioactive peptides, extracted from *Manduca* CNS. Both compounds cause cardioacceleration (Fig 2 B). However, perfusion of 2-bromolysergic acid diethylamide (BOL), which itself only slightly affects heart rate, completely abolishes the response to 5HT (Fig 2 C.I). BOL acts as a specific 5HT receptor blocker (Platt and Reynolds 1985) but has no blocking effect on the hearts
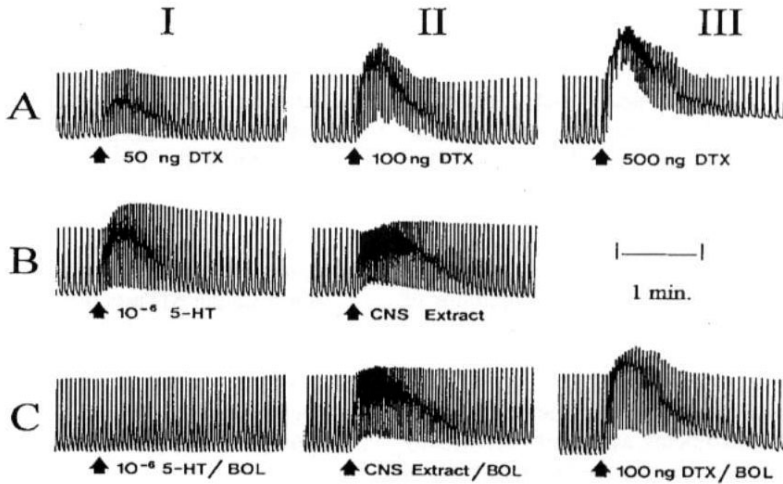
Figure 2. The effects of Destruxin A and other test substances on the frequency of *Manduca sexta* heart beat (addition of test substance indicated by the arrows). A=The effect of increasing concentrations of DTX A on the frequency of *M. sexta* heart beat (A.I=50 ng DTX; A.II=100 ng DTX; A.III=500 ng DTX); B.I=Cardioactive effect of 10^{-6} M 5-HT; B.II:=Cardioactive effect of CNS Extract; C.I=Cardioactive effects of 5-HT were blocked by 10^{-4} M 2-bromolysergic acid diethylamide (BOL) perfused continuously for 2h before addition of 5-HT; C.II=Cardioactive effect of CNS Extract was not blocked by BOL; C.III=Cardioactive effect of DTX A was not blocked by BOL; note: The time bar represents a one minute period.

response to CNS extract or DTX A (Fig 2 C. II, III). Therefore, DTX A must act at a site or sites other than 5HT receptors. Although it seems unlikely that DTXs act at the same receptors as the cardioactive peptides extracted from the CNS, this possibility cannot
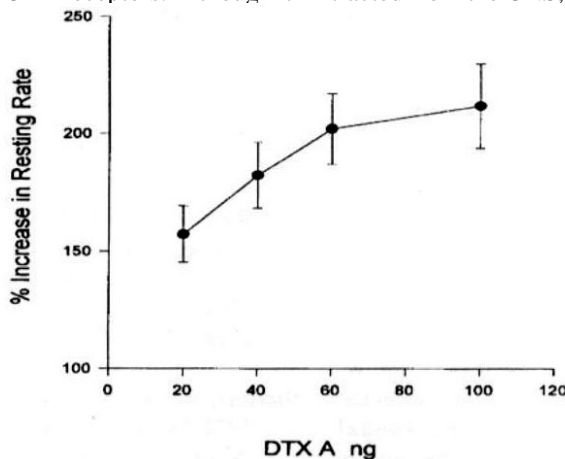

Figure 3. Dose response curve of percentage increase in resting rate of *Manduca sexta* heart beat with increasing doses of destruxin A; note: Results are means ($\pm$ SD) of three replicate applications of destruxin using the same heart preparation.

be ruled out at present.

Several of the physiological actions of DTXs on insect cells, most notably the depolarization of lepidopteran muscle membranes (Samuels *et al*. 1988b), are related to extracellular Ca^{2+}. Further neurophysiological studies suggested that DTXs activate a voltage-dependent calcium channel (Bradfisch and Harmer 1990). However, a recent study has been shown that DTX B acts as a specific, dose-dependent and reversable inhibitor of vacular-type ATPase, which maintains acidic homeostasis in membrane-bound organelles in eukaryotic cells (Muroi *et al*. 1994). DTX B was found to block acidification of intracellular compartments, a pivotal event in many aspects of cell physiology, which may account for most, if not all, of the effects of this toxin. It is very likely that DTX A also acts as an inhibitor of specific ATPase, which may result in acceleration of *Manduca* heartbeat in response to pH changes within the muscle cells.

Acknowledgements

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