Hirsutellin A, a toxic protein produced in vitro by Hirsutella thompsonii

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A toxic protein, hirsutellin A, has been purified from the mitc fungal pathogen, Hirsutella thompsonii, using ammonium sulphate precipitation, ion exchange chromatography and gel filtration on Bio-Gel P-10. The protein has been characterized as a monomer with a molecular mass of 15 kDa and an isoelectric point of 10.5. The amino acid composition and the N-terminal sequence of hirsutellin A (34 amino acids) have been determined. From these results, the toxin appears to be distinct from other known proteins. It is not glycosylated, and does not show proteolytic activity. The toxin is also antigenic, thermostable and not inactivated by treatments with proteolytic enzymes. Toxicity bioassays showed that injection of larvae of the waxmoth, Galleria mellonella, with hirsutellin A at low dosages [1 \( \mu \)g toxin (g body wt\(^{-1}\)] caused a high mortality rate. Hirsutellin A was also toxic per os to neonatal larvae of the mosquito Aedes aegypti.

Keywords: Hirsutella thompsonii, hirsutellin A, toxic protein, insecticide, acaricide

INTRODUCTION

Hirsutella thompsonii is a specific fungal pathogen of Acarina, particularly eriophyid and tetranychid mites, inhabiting citrus and other plants in most subtropical and tropical regions (Samson et al., 1980). This fungus causes regular natural epizootics in populations of the citrus rust mite, Phyllocoptruta oleivora, in Florida and has been developed as a microbial insecticide for use against this mite (McCoy, 1981; McCoy & Couch, 1982). Recently, Vey et al. (1993) have shown that culture filtrates of H. thompsonii were toxic to Galleria mellonella larvae and Drosophila melanogaster adults via injection and per os application, respectively. The biologically active compounds were not identified. Many entomopathogenic fungi produce toxic metabolites in artificial culture and, in a few cases, in vivo (Suzuki et al., 1971; Vey et al., 1986).

Most of the fungal toxins described have been low molecular mass cyclic peptides. For example, the fungal pathogen Metarhizium anisopliae has been shown to produce several cyclodepsipeptides, the destruxins, which are toxic to insects (Roberts, 1969; Suzuki et al., 1970). Insecticidal cyclic peptides such as beauvericin and bassianolide have been isolated from another myco-pathogen, Beauveria bassiana (Hamill et al., 1969; Suzuki et al., 1977). Injection of these toxic peptides into G. mellonella larvae induced immediate paralysis and quick mortality (Roberts, 1966; Suzuki et al., 1977). Symptoms of toxicity caused by H. thompsonii culture filtrates were different as no paralysis and a slow death were observed in challenged G. mellonella larvae (Vey et al., 1993).

This study was carried out to identify the toxic metabolites detected in culture filtrates of H. thompsonii. We have purified and characterized one of these toxic compounds and studied its insecticidal activity. This is the first report of the purification of a toxin from H. thompsonii.

METHODS

Preparation of fungal cultures and filtrates. Hirsutella thompsonii var. thompsonii strain HTF 87, originally isolated from the citrus rust mite Phyllocoptruta oleivora in 1987, was obtained from the culture collection of C. W. McCoy at the University of Florida. Using methods described previously (Vey et al., 1993), slant cultures were produced on soil fungus medium (SFM). Conidia were inoculated into 35 ml of Czapek-Dox broth plus 1% (w/v) yeast extract in a 100 ml Erlenmeyer flask and cultured at 25°C on a rotary shaker at 110 r.p.m. for 7 d as a primary culture. For large-scale growth of the fungus, 2 ml of the primary culture were transferred into 350 ml of the same medium in a 1 1 Erlenmeyer flask and cultured in the same way for 7 d before harvesting by filtration through cheesecloth and filter paper to remove mycelial material. The mycelial biomass was dried in an oven at 60°C for 24 h and then weighed.

Assays for toxicity. Toxicity was tested on sixth instar Galleria mellonella larvae (mean weight 200 ± 16 mg) reared as described previously (Vey et al., 1993). After sterilization through a 0.45 \( \mu \)m filter, 8 \( \mu \)l of either culture filtrate or chromatographic fractions were injected into the haemocoel through a proleg of
each larva using a Desaga microinjector. Control larvae were injected with either uninoculated broth or buffers and salt solutions used for the fractionation. For each treatment, three replicates of 20 larvae were tested. Larval mortality was recorded daily. For assays on mosquitoes, neonatal larvae of *Aedes aegypti* (strain Ngoye from Senegal) were inoculated into wells of 96 well microtitre plates containing 200 µl of test solutions. For each treatment, three replicates of 24 larvae were tested. Larval mortality was evaluated at 24, 48, and 72 h post-inoculation.

**Purification procedure.** Proteins from the crude filtrate, precipitated with 90% saturation of ammonium sulphate, were collected by centrifugation at 12000 g for 1 h. The precipitate was dissolved in a minimum volume of distilled water (1/30 to 1/40 of the volume of filtrate) and desalted through a Sephadex G-25 (Pharmacia) column (2.5 x 30 cm) in 50 mM Tris/HCl buffer, pH 8.0. The desalted fraction was applied to a DEAE-Trisacryl (IBF) column (2.5 x 16 cm) pre-equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The column was washed and then bound material was eluted with 1 M NaCl in the same buffer. The DEAE fraction containing unbound material was dialysed against 50 mM sodium acetate buffer, pH 5.0, and further resolved by CM-Trisacryl (IBF) ion exchange chromatography (2.5 x 16 cm column in 50 mM sodium acetate buffer, pH 5.0) with 0.1 M NaCl followed by a 300 ml linear gradient of 0.2-0.4 M NaCl in the same buffer. The flow rate was about 140 ml h⁻¹ and 7 ml fractions were collected for ion exchange chromatography. Finally, the active CM fraction concentrated with 90% ethanol precipitated with 90% ethanol and creatinase as non-glycosylated control protein.

**Determination of total protein concentration.** Total protein concentration was determined with the Bio-Rad Protein Assay method based on the method of Bradford (1976). Bovine serum albumin was used as the standard.

**Electrophoresis.** Protein samples from the different fractionation steps were analysed by SDS-PAGE with 20% (v/v) acrylamide gels and the buffer system described by Laemmli (1970). Low molecular mass standards (Sigma) were run concurrently and protein bands visualized by either Coomassie blue R-250 or silver staining using the Bio-Rad Silver Stain kit.

**Isoelectric point determination.** The isoelectric point of the pure toxin was determined by polyacrylamide gel isoelectric focusing performed with 125 x 125 x 0.3 mm Servalyt Precotes pH 3-10 (Serva) on a Pharmacia FBE 3000 apparatus. Gels were loaded with Protein Test Mixture 9 (Serva) as standard and stained with Coomassie blue R-250.

**Amino acid and N-terminal sequence analysis.** The isolated toxic protein was hydrolysed with 6 M HCl in sealed tubes under vacuum for 24 h at 110 °C. The composition of the amino acids in the hydrolysate was determined with a Beckman 7300 amino acid analyser. No corrections for incomplete hydrolysis or for decomposition were made, and amounts of tryptophan and cysteine were not determined. The N-terminal sequence of the toxin was determined by automated Edman degradation using the gas-phase technique in an Applied Biosystems 470 A sequenator. PTH-amino acids were identified by HPLC.

**Proteases and temperature effects.** Solutions of pure toxin at 100 µg ml⁻¹ were incubated overnight at room temperature with solutions of trypsin (Difco) at 1 mg ml⁻¹, protease type VI from *Streptomyces griseus* (Sigma) at 1 mg ml⁻¹, Proteinase K (Boehringer) at 100 µg ml⁻¹ and dispase (neutral protease) from *Bacillus polymyxa* (Boehringer) at 100 µg ml⁻¹. Additional samples of toxin were incubated 30 min at 100 °C and 90 min at 60 °C.

**Glycan analysis.** The carbohydrate composition of the toxin was examined with the DIG Glycan Detection Kit (Boehringer) according to the protocols of Haselbeck & Hösel (1990). The pure toxin was also incubated with 0.4 and 1 unit of N-glycosidase F (Boehringer) according to the method of Haselbeck & Hösel (1988) and then analysed by SDSPAGE. For both assays, transferrin was used as control glycoprotein and creatinase as non-glycosylated control protein.

**Determination of enzyme activity.** The enzyme activity of the pure toxin was examined with the API ZYM semi-quantitative micromethod system according to the manufacturer’s instructions. Each microtitre plate containing Chromogenic enzyme substrates was incubated with 65 µl of pure toxin at 100 µg ml⁻¹.

**Polynodal antibody production and Western blotting.** A rabbit was immunized with a solution of native hirsutellin A according to the method of Sambrook *et al.* (1989) and the antiserum stored at −20 °C until needed. The presence of specific-toxin antibody in the serum was determined by Western blotting. Protein samples of chromatographic fractions were partitioned in 20% (w/v) SDS-PAGE gels and transferred electrophoretically to nitrocellulose BA 85 membranes (Schleicher & Schuell) using 25 mM Tris/HCl (pH 8.6), 192 mM glycine, 20% (v/v) methanol. The efficiency of protein transfer was evaluated by prestaining Western blots with Ponceau red. Membranes, blocked with 5% milk in 50 mM Tris/HCl (pH 7.4), 200 mM NaCl, 0.05% Tween 20, were incubated with either control rabbit serum or anti-hirsutellin A rabbit serum (diluted 1:500) for 2 h at 37 °C followed by incubation in goat-anti-rabbit IgG–peroxidase conjugate (Sigma).

**RESULTS**

**Purification of hirsutellin A**

The toxic metabolites in the crude filtrate of *H. thompsonii* were retained by dialysis (molecular mass cut-off of 6–8 kDa), were precipitated with ammonium sulphate and...
were excluded from a Sephadex G-25 column. These results suggested that the mycotoxins were high molecular mass compounds and were purified them accordingly as described in Methods. The first peak eluted from the DEAE-Trisacryl column with 50 mM Tris buffer was toxic when injected to G. mellonella larvae. Larvae demonstrated characteristic symptoms, becoming swollen and flaccid after exposure to this fraction. The second peak eluted with high-salt buffer was also toxic to G. mellonella. These larvae were not swollen but developed a brown coloration over the cuticle. The first peak containing unbound material produced five peaks on a CM-Trisacryl column (Fig. 1). Peak V eluted with 0.3 M NaCl was toxic to test insects. The active CM fraction applied to the gel permeation column of Bio-Gel P-10 produced a single peak containing toxic activity. Analysis of the various chromatographic fractions by SDS-PAGE followed by silver staining revealed the presence of a single peptide band at the last purification step (Fig. 2). The yield of the pure product, hirsutellin A, was 0.035 mg pure toxin (g dry mycelium wt)\(^{-1}\).

**Characterization of hirsutellin A**

The behaviour of hirsutellin A on polyacrylamide gels under denaturing conditions suggested a single polypeptide chain with molecular mass of about 15 kDa. By gel filtration on the Bio-Gel P-10 column calibrated with proteins of known molecular mass, the molecular mass of hirsutellin A was determined as 12 kDa (data not shown). This result indicated that the native hirsutellin A comprised only one copy of the peptide chain. Analysis of hirsutellin A by isoelectric focusing showed a single protein band with the apparent isoelectric point of 10.5. No sugars could be detected in hirsutellin A with the methods used. None of the 19 enzyme activities detectable by the API ZYM system, including phosphatases, esterases, aminopeptidases, proteases with trypsin and chymotrypsin activities and glucosidases, were observed with the purified hirsutellin A.

The amino acid composition of hirsutellin A is listed in Table 1. Several features of its composition must be emphasized. A high proportion of lysine is responsible for the basic property of hirsutellin A. Additionally, this toxin contained a high percentage of the hydrophilic amino acids, aspartic acid (+asparagine), glutamic acid (+glutamine) and lysine, which may explain its solubility in the various buffers and in water. Glycine and alanine were present in high proportion. Furthermore, in SDS-PAGE without β-mercaptoethanol, hirsutellin A migrated faster than under denaturing conditions (Fig. 2). This result suggested that hirsutellin A contained at least two cysteines involved in the formation of an intrachain disulphide bridge. The N-terminal amino acid was not blocked and the N-terminal sequence (34 amino acid residues) was determined to be APKVTSPKLDGRE-KPKVVDATAQASKAGLT. The generated N-terminal sequence was compared to five component databases (PDB, SwissProt, PIR, Spudate, Genpept)
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Fig. 3. Western blot of the purification steps of hirsutellin A probed with anti-hirsutellin A rabbit polyclonal IgG at a dilution of 1:500 then incubated in goat-anti-rabbit IgG-peroxidase conjugate. Lanes: 1, Sephadex G-25 pool; 2, DEAE buffer pool; 3, CM peak V pool; 4, Bio-Gel P-10 pool. Note that only the 15 kDa band indicated by the arrow was intensely stained.

using the BLAST network service. No significant homology to any other known protein was revealed.

Repeated injection of pure hirsutellin A into rabbits has produced specific anti-toxin antibody. On Western blots of SDS gels of chromatographic fractions, only the 15 kDa band gave an intense positive staining reaction to the polyclonal serum used at a dilution of 1 in 500, even in the wells of non-purified samples containing other fungal proteins (Fig. 3). There was no staining of blots probed with control rabbit serum.

To test the stability of hirsutellin A its toxicity to G. mellonella larvae after various treatments was studied. The activity was unchanged after incubation for 30 min at 100 °C and 90 min at 60 °C. Freezing and thawing, as well as freeze-drying, did not affect the bioactivity. Treatment with trypsin, protease VI, proteinase K and dispase did not cause a decrease in toxicity.

Insecticidal activity of hirsutellin A

Injection of G. mellonella larvae with 25, 50 and 100 µg hirsutellin A ml⁻¹, equivalent to 1, 2 and 4 µg protein (g body wt)⁻¹ caused 100% mortality (Table 2). However, the mortality occurred later as the concentration of hirsutellin A decreased. The mean lethal time was 8.8 d at 100 µg ml⁻¹, 17.1 d at 50 µg ml⁻¹, and 24.2 d at 25 µg ml⁻¹. At this last concentration, treated larvae died as pupae as or as pharate adults before emergence from the pupae. No mortality was observed at 12.5 µg ml⁻¹, nor in control larvae injected with water. Hirsutellin A was slow-acting, since at the highest concentration assayed, complete mortality was not expressed until 15 d post-injection.

Purified hirsutellin A was toxic per os to neonatal A. aegypti larvae in microtitre plate bioassays. Larval mortality increased with an increase in toxin dosage (Table 3). Low larval mortality was detected at 5 and 10 µg ml⁻¹. A concentration of 15 µg ml⁻¹ caused 58.3% mortality to larvae at 72 h post-inoculation, whereas at 20 µg ml⁻¹, larval mortality reached 97.9% at 48 h and 100% at 72 h post-inoculation. Mortality of control larvae incubated in water was less than 5%.

DISCUSSION

Research on toxins from entomogenous fungi is motivated by the importance of the analysis of the role of these microbial metabolites as chemical messages involved in fungal pathogenesis. An additional aim, from an applied point of view, is the evaluation of their potential as new bioinsecticides. In this paper, we describe the purification and characterization of hirsutellin A, a toxic metabolite from H. thompsonii, a fungus pathogenic for mites which was the basis of the first registered commercial mycopesticide in the USA.

Our investigations have demonstrated that the toxic activity detected in culture filtrates of H. thompsonii by Vey et al. (1993) was not due to low molecular mass metabolites such as cyclopeptides, organic acids or pigments which are known to be produced by various other fungi pathogenic for invertebrates (Lysenko & Kučera, 1971; Roberts, 1981; Khachatourians, 1991). The biological activity was exclusively detected in high molecular mass fractions after gel filtration on Sephadex G-25 and dialysis experiments. Furthermore, extraction of filtrates with conventional organic solvents used to extract cyclic peptides did not produce active fractions (data not shown). It has been demonstrated that the toxicity was caused by at least two high molecular mass proteins, one adsorbed to the DEAE-Trisacryl column and the second present in the initial fraction eluted with the Tris buffer. Our efforts have been directed at purifying and characterizing the toxin, hirsutellin A, not bound to the anion exchanger using conventional chromatographic methods. The biochemical properties demonstrated that hirsutellin A is a novel and original molecule different from previously described toxic proteins of entomogenous fungi. Thus, Kučera & Samsinakova (1968) partially purified high and low molecular mass proteases from B. bassiana which were toxic to G. mellonella larvae. The studies of Kučera (1980) revealed the production of two toxic proteases by M. anisopliae with respective molecular masses of 35 and 71 kDa. In both cases, the toxic action was associated to proteolytic activities whereas no peptidase or protease activity was observed with hirsutellin A.
Toxic protein of the fungus *Hirsutella*

**Table 2.** Toxicity of hirsutellin A to *Galleria mellonella* larvae

Larvae were injected with 8 μl of either toxic solutions or water as control. Three replicates of 20 larvae were used for each treatment. No mortality was observed for control larvae.

<table>
<thead>
<tr>
<th>Hirsutellin A (μg ml⁻¹)</th>
<th>Percentage mortality*</th>
<th>Mean lethal time (d)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 10 15 20 25 30</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0 0 0 0 0 0</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0 0 6.6±5.7 40±17.3</td>
<td>733±21.6 100 24.2±2.0</td>
</tr>
<tr>
<td>50</td>
<td>7.5±5 35±17.3 41.2±22.1</td>
<td>80±8.1 100 17.1±1.8</td>
</tr>
<tr>
<td>100</td>
<td>18±9.5 63±20.4 100</td>
<td>8.8±1.4</td>
</tr>
</tbody>
</table>

* Mean value ± SD after time (d) indicated.

**Table 3.** *Per os* toxicity of hirsutellin A to *Aedes aegypti* larvae

Neonatal larvae were incubated in 200 μl of either toxic solutions or water as control. Three replicates of 24 larvae were used for each treatment.

<table>
<thead>
<tr>
<th>Hirsutellin A (μg ml⁻¹)</th>
<th>Percentage mortality*</th>
<th>24 48 72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>0 0 2±2.9</td>
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</tr>
<tr>
<td>5</td>
<td>0 0 4±1</td>
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</tr>
<tr>
<td>10</td>
<td>0 6.2±2.9 10.6±2.7</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>4.1 27±2.9 58.3±5.8</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>80.9±14.2 97.9±21</td>
<td>100</td>
</tr>
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* Mean value ± SD after time (h) indicated.

as suggested by results of a semiquantitative enzyme method.

Experimental bioassays using *G. mellonella* larvae and neonatal *A. aegypti* larvae demonstrated that the insecticidal activity of hirsutellin A could be expressed via injection or per os absorption. Vey et al. (1993) have also reported a *per os* toxicity of crude filtrates of *H. thompsonii* to *D. melanogaster* adults. In addition, the activity of hirsutellin A was evaluated against the mite *P. oleivora*, the original host of *H. thompsonii*. Significant mite mortality (100%), possibly resulting from both contact and *per os* activities, was observed 48 h after exposure to toxin preparations (C. W. McCoy, C. Omotto, I. Mazet & A. Vey, unpublished data).

To elucidate the role of toxins in pathogenesis of fungal infections, it is essential to know by which mechanisms these compounds interact with host tissues and cells. If previous studies have revealed a strong cytotoxic effect of *H. thompsonii* culture filtrates in the experimental insect host, *G. mellonella* and in invertebrate cell cultures (Vey et al., 1993), the site and mode of action of hirsutellin A at the cellular and subcellular level remain presently unknown. Toxic proteins produced by moulds of the genus *Aspergillus* such as α-sarcin isolated from *A. giganteus* (Sacco et al., 1983) and restrictocin and mitogillin isolated from *A. restrictus* (Lopez-Otin et al., 1984; Fernandez-Luna et al., 1985) are similar to hirsutellin A, being basic and having a molecular mass of about 17 kDa. These proteins inhibit protein synthesis by inactivating ribosomes (Chan et al., 1983; Fando et al., 1985). It would be of interest to compare hirsutellin A with these toxins on the basis of their full amino acid sequence and to study the effect of hirsutellin A on protein synthesis.

The success of this purification and characterization of hirsutellin A has allowed research on the biochemical aspects of the mode of action of this molecule to be initiated, as well as investigations at the level of the corresponding gene, the first step being to clone and sequence the gene. By using the specific antiserum, it should be possible to determine if the toxin is produced in vivo and to establish if it has any role in pathogenesis. Moreover, the biological activities of hirsutellin A on different insect and acarine hosts, as well as its stability, suggest that this fungal molecule could represent a new model for bioinsecticide development.

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**REFERENCES**


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