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Proteolytic activity and antibiotic production by *Trichoderma harzianum* in relation to pathogenicity to insects

Joe Shakeri, Howard A. Foster *

Biomedical Sciences Research Institute, School of Environment and Life Sciences, University of Salford, Salford, Greater Manchester M5 4WT, UK

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Abstract

Two strains of *Trichoderma harzianum*, 101645, an insect pathogen and 206040, used for biological control of fungal plant pathogens were investigated for the production of serine protease, chitinase and antibiotic activity in relation to entomopathogenicity. Both strains produced serine protease with a M_r of 31 kDa and chitinase with a M_r of 44 kDa. Enzymes from both strains had similar characteristics and were produced during the growth phase. Both strains also produced peptaibols active against fungi in late growth and stationary phases which differed in their amino-alcohol content. The peptaibols were insecticidal when fed to larvae of *Tenebrio molitor* or when applied to the cuticle together with the serine protease. The results suggest that the virulence factors involved in biocontrol are the same as those for insect pathogenicity. This may affect the use of *Trichoderma* spp. for biocontrol as there may be effects on non-target insect species.

Keywords: Biological control; Chitinase; Entomopathogen; Peptaibol; Protease; *Trichoderma harzianum*

1. Introduction

Mycoparasitic fungi of the genus *Trichoderma*, particularly *Trichoderma harzianum*, have been used for biocontrol of pathogens of crop plants both soil-borne, e.g. *Rhizoctonia solani* and *Sclerotium rolfsii* [1,2], and foliar pathogens including *Botrytis cinerea* on cucumber [3]. *Trichoderma* pellets (Binab-T) injected into the trunks of trees have proven to be effective in the treatment of silver leaf disease of fruit trees caused by *Chondrostereum purpureum* [4]. Possible mechanisms involved in *Trichoderma* antagonism are induced resistance, antibiosis, competition and mycoparasitism. *Trichoderma* spp. have been shown to produce glucanases [5], protease [3,6], chitinase [7] and a range of non-volatile and volatile antibiotics active against a range of fungi [8,9]. The cell-wall degrading enzymes protease and chitinase are induced in *Trichoderma* during the parasitic interaction [10]. The protease (prb1) has been purified and biochemically characterised from *T. harzianum* [6]. The biocontrol activity was improved by over expression of prb1 [11].

Fungi can also be entomopathogenic and the mode of entry is usually by penetration of host cuticle by a combination of enzymatic degradation, principally by protease and chitinase enzymes and mechanical pressure by haustorium production [12]. One of the main pathogenicity determinants for *Metarhizium anisopliae* against *Manduca sexta* larvae is an extracellular subtilisin-like chymoelastase designated Pr1 and a similar enzyme has been identified in other entomopathogenic fungi [13].

One potential problem, which may affect the acceptance of *Trichoderma* spp. as useful control agents, is the possibility of activity against non-target species. Because Pr1 from insect pathogens has similar properties to prb1 from *Trichoderma* spp. it is possible that the proteinases may play a key role in both entomopathogenicity and antifungal action. Indeed larvicidal activity of *T. harzianum* IMI 206040 against the elm bark beetle *Scolytus* spp. have been reported [14] and *T. harzianum* IMI 101645 is also catalogued as being entomopathogenic [15]. We therefore examined the differences between *T. harzianum* 101645, an insect pathogen and 206040, a biocontrol strain used in Binab-T pellets. In this report we show that both have activity on the larvae of the mealworm *Tenebrio obscurus*, both produce peptaibol antibiotics, serine protease and chitinase. Possible mechanisms of pathogenesis are discussed.

* Corresponding author. Tel.: +44 161 295 3832; fax: +44 161 295 5015.
E-mail address: h.a.foster@salford.ac.uk (H.A. Foster).

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2. Materials and methods

2.1. Organisms and culture conditions

Two strains of *T. harzianum* (IMI 206040 and IMI 101645) were used in this study, *B. cinerea* strains 448 and 452, *Ophiostoma novo-ulmi*, *Thanophorus cucumeris* 305038, *Fusarium culmurium*, *Fusarium oxysporum*, *Penicillium notatum*, *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Chromobacterium lividum* and *Micrococcus luteus* were obtained from the Biosciences Research Institute, University of Salford. Fungi were cultured on Sabouraud Dextrose agar (SDA; Oxoid, Basingstoke, Hants, UK) slants, incubated at 25 °C and bacteria on nutrient agar (Lab M, Bury, UK) at 35 °C and then stored at 5 °C.

Cell-culture was carried out using a Series 2000 Bioreactor (L.H. Engineering, Slough, Bucks) with 8 l of Sabauroud Dextrose medium for antibiotic production or Czapek-Dox medium (LabM) for enzyme production in a 16 l vessel at 25 °C, an airflow rate of 10 l min^{-1} and an agitation speed of 200 rpm. The medium was inoculated with 400 ml of 3-day-old culture of *T. harzianum* incubated on an orbital shaker at 25 °C and 180 rpm in the same medium. The pH was controlled at 6.0 for proteinase production and 7.0 for antibiotic production using 0.1N NaOH and 0.1N HCl. The growth yield was estimated by turbidity measurement at 600 nm and by cell dry weight determinations. Samples were removed at intervals and enzyme and antibiotic activities determined. For routine preparations, cultures were incubated for 5 d, cooled to 5 °C then centrifuged in a Sharples Super Centrifuge rotor type MV 41–244 (Pennwalt Sharples, Camberley, Surrey, UK) at 25,000 rpm. The supernatant was kept at 5 °C for extraction of antibiotics or enzymes.

2.2. Induction of enzyme activity by different substrates

Ehrlenmeyer flasks (250 ml) containing 150 ml CD with 1% different carbon sources (glucose, crab cuticle chitin, casein, locust cuticle powder; all from Sigma–Aldrich) and *B. cinerea* cell-wall preparation (*B. cinerea* grown in CD for 3 d then filtered, washed ×3 with distilled water, dried and ground in a pestle and mortar to a fine powder) were adjusted to pH 6.8, autoclaved, inoculated with 5 mm diameter discs of 3-day-old culture of *T. harzianum* and incubated at 25 °C. Samples were removed after 7 d and assayed for enzyme activity and protein concentrations.

2.3. Assay for antibiotic activity

The antimicrobial activity of the crude extract and the partially purified antibiotic (s) were determined using 10 mm diameter discs of Whatman No. 1 filter paper impregnated with antibiotic agent according to the method of Loo et al. [16]. An arbitrary calibration curve with zone diameter against increasing concentrations of crude antibiotic extract was prepared using *B. cinerea* 448. The diameter of the zone of activity was measured and the diameter of the disc subtracted. One unit of antibiotic activity was defined as giving a 2 mm dia zone of inhibition. Volatile antibiotics were detected using the sandwich plate method of Dennis and Webster [17] on SDA.

2.4. Ethyl acetate extraction

Antifungal activity was extracted from the culture supernatant with ethyl acetate after Okuda et al. [18]. Culture filtrate was mixed with one-quarter volume of ethyl acetate, shaken for 15 min and the mixture allowed to separate for 30 min. The upper phase (ethyl acetate) was separated from the exhausted supernatant (lower phase). The solvent phase was concentrated under reduced pressure by rotary evaporation at 30 °C, dissolved in ethyl acetate (1 ml) and fractionated on TLC plates (silica gel; 20 cm × 20 cm) with chloroform:methanol:acetic acid:water 65:25:4:3. The plates were dried at room temperature, and the active fractions identified by bioautography [19] using *B. cinerea* as test organism. The active spots were scraped off, eluted with methanol, filtered, dried and dissolved in methanol–water 86:14. The preparation was fractionated by preparative HPLC (Perkin-Elmer 3B) using a silica gel column and eluted with methanol:water 86:14 with detection at 210 nm. Active fractions were pooled and methanol was removed in a stream of dry nitrogen. The dried fractions were kept at 5 °C.

2.5. Chemical analysis of antifungal agents

2.5.1. Hydrolysis

The dried antifungal agents (1 mg) were suspended in 500 μl 6N HCl in a heavy walled vial (1 mm) with Teflon-lined screw caps and heated at 100 °C for 24 h. The hydrolysates were evaporated to dryness in a stream of nitrogen at 40 °C.

2.5.2. Thin layer chromatography

Samples of the hydrolysed purified antifungal agents were dissolved in 0.1N HCl (5 μl) and were chromatographed on silica gel TLC plates, using chloroform–methanol–water–acetic acid (65:25:4:3) as the solvent system. The plates were dried for 5 min at 100 °C in the oven to remove excess solvent from the plates. The plates were sprayed with ninhydrin (10 g l^{-1} in ethanol) to visualise the amino acids, dried for 10 min and the spots developed by heating at 100 °C for 10 min. R_f values of the samples were measured and compared with amino acid standards.

2.5.3. Gas chromatography–mass spectrometry

Purified antibiotic hydrolyzates (1 mg) and amino acid standards (1 μg) were dissolved in 0.5 ml of 2.5N HCl in 1-propanol and heated at 100 °C for 1 h. Excess reagents were removed in a stream of dry nitrogen. Dichloromethane (200 μl) and 50 μl pentafluoropropionic anhydride were added and the mixtures heated at 100 °C for 20 min. Reagents were then removed in a stream of nitrogen and the residues dissolved in 200 μl dichloromethane. Samples were injected splitless onto a 25 m × 25 mm fused silica column on a Perkin-Elmer Tr10 1000 Mass spectrometer (Perkin-Elmer, Wellesley, MA) with a scan range of 45–650 amu. The carrier gas was nitrogen at 1 ml min^{-1} and the chromatograph was programmed from an initial temperature of 80 to 200 °C at 10 °C min^{-1}.

2.6. Ultra-violet spectrometry

Partially purified antibiotic agent was resuspended in analytical grade methanol. Ultra-violet spectra were measured in a Unicam-SP-800A spectrophotometer (Unicam-Thermo Spectronic, Rochester, NY) with a quartz cell of 10 mm path length.

2.7. Measurement of enzyme activities

2.7.1. Total protease

Total proteolytic activity was measured using hide powder azure (HPA; Rinderknecht et al. [20]). HPA (20 mg) was suspended in 4.5 ml 50 mM phosphate buffer pH 7.4 and 0.5 ml of enzyme solution was added. The tubes were incubated at 37 °C for 1 h with occasional agitation. The mixture was filtered through Whatman No. 50 filter paper to remove excess substrate and the A_{595} determined. A calibration curve was prepared by total hydrolysis of known amounts of substrate (0–12 mg) with trypsin (Sigma; 1 mg ml^{-1}). One unit of protease activity was defined as being equivalent to 1 mg substrate hydrolysed per ml of sample in 1 h at 37 °C.

2.7.2. Chitinase

Chitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin [7]. A colloidal suspension of chitin (5 mg ml^{-1} in 50 mM phosphate buffer at pH 6.30: 2 ml) was mixed gently with 1 ml purified chitinase and made up to a final volume of 5 ml with phosphate buffer. The turbidity of the mixture was measured at 420 nm immediately and after incubation at 37 °C for 20 min. One unit of chitinase activity was the amount of enzyme which brought about a decrease of 0.01 optical density units per minute.

2.7.3. Basic proteinase

Enzyme was dissolved in 50 mM l^{-1} sodium phosphate buffer pH 7.4 (PB). The substrate solution (10 mM l^{-1}) was prepared by dissolving succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (pNA) in 1 ml of 10 mM dimethylsulphoxide and diluting with PB containing calcium acetate (0.02 M l^{-1}). Substrate solution (1 ml) was added to test tubes, 1 ml of enzyme solution added and the total volume adjusted to 5 ml with PB. The mixtures were incubated at