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Identification and characterization of an insect toxin protein, Bb70p, from the entomopathogenic fungus, *Beauveria bassiana*, using *Galleria mellonella* as a model system

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ABSTRACT

An insect-toxic protein, Bb70p, was purified from *Beauveria bassiana* 70 using ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. Bb70p has a high affinity for anion exchangers and 2D electrophoresis results revealed a single spot with a molecular weight of 35.5 kDa and an isoelectric point of ~4.5. Bb70p remains active from 4 to 60 °C, within a pH range of 4–10, but is more active in slightly acidic pH. A pure protein, Bb70p does not have any carbohydrate side chains. The protein caused high mortality by intra-haemocelic injection into *Galleria mellonella* with LD50 of 334.4 μg/g body weight and activates the phenol oxidase cascade. With a partial amino acid sequence comparison using the NCBI database, we showed no homology to known toxin proteins of entomopathogenic fungi. Thus, Bb70p appears to be an insect toxin protein, demonstrating novelty. Identification of this insect-toxic protein presents potential to enhance the virulence of *B. bassiana* through genetic manipulation.
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1. Introduction

The entomopathogenic fungus *Beauveria bassiana* is an important and cosmopolitan microbial agent used to control many important pests (Charnley and Collins, 2007; Faria and Wraight, 2008). About 170 years ago, *B. bassiana* (Vuilemin) was described for the first time as a biocontrol agent to control insects (Zimmermann, 2007). Entomopathogenic fungi have been used extensively due to their pathogenicity and broad host range (Butt, 2002; Fan et al., 2007; Thomas and Read, 2007).

Although entomopathogenic fungi are used to control a variety of insect pests, they still do not fulfill the criteria for commercialization, primarily due to their slow mortality rate (St. Leger and Wang, 2009). Hence, the use of entomopathogenic fungi could be increased, if the killing efficiency is improved. This could be achieved in a number of ways, including enhancing the fungal toxin production. It has been suggested that genetic modification could be used to enhance this ability (Hegedus and Khachatourians, 1995; St. Leger and Screen, 2001). This requires research to be focused on the identification and development of bioactive metabolites which have more rapid effects, similar to chemical pesticides.

It has been demonstrated that a number of entomopathogenic fungi secrete insecticidal, anti-feedant, or toxic bioactive compounds in liquid cultures which can be purified (Quesada-Moraga et al., 2006a,b). For example, Spinosad and Abamectin are two widely used commercial insecticides based on the culturing, harvesting, and purification of microbial metabolites (Copping and Menn, 2000; Godfrey et al., 2005; Mazet et al., 1994; Vey, 1998; Vey et al., 2001). Many of the studies to date have been carried out on *B. bassiana* regarding the identification of cuticle degrading determinants and enzymes such as subtilisins and metalloproteases (Charnley, 1989; Charnley and St. Leger, 1991; Saint-Leger, 1993; Khachatourians, 1996; Griesch and Vilcinskas, 1998; Shimizu et al., 1993a,b). Less attention has been given to post-penetration events, particularly the release of soluble toxic components in the haemolymph. Very little is known about the secretion of bioactive metabolites by *B. bassiana in vivo* during the infection process. However, in a liquid culture, *B. bassiana* produces cyclic

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peptides of low molecular weight such as beauvericin, enniatins, and bassianolide, which possesses insecticidal properties (Roberts, 1981; Vey et al., 2001). Similarly, a red-colored dibenzoquinone, Oosporein is produced by the *Beauveria* species. Cyclosporins A and C have been detected in *B. bassiana* mycelium and are active against mosquito larvae (Weiser and Matha, 1988; Weiser et al., 1989). Kucera and Samsinakova (1968) reported that macromolecules, having protease activity, isolated from *B. bassiana*, were toxic on injection into *Galleria mellonella* larvae. This idea of a novel toxic factor was also recently advanced by Quesada-Moraga and Vey (2003, 2004) who demonstrated that filtrates of *B. bassiana* (isolated from the locust *Dociostaurus maroccanus*), secretes a novel protein of 60 kDa, bassiacridin, which is specifically toxic on injection into *Locusta migratoria* nymphs.

In this study, we report on the purification and characterization of a toxin protein from the entomopathogenic fungus, *B. bassiana* sensu lato (Rehner et al., 2011), isolate *B. bassiana* 70 (hereafter referred to as *B. bassiana* 70), *in vitro*. Our results will be helpful in developing fungal-filtrate based biopesticides which will be biological in origin and provide rapid control of insect pests. In addition, this research offers the potential to develop more virulent entomopathogenic fungal strains through genetic manipulation. This will, consequently, increase the prospects for commercialization of more environmentally-friendly biopesticides.

2. Material and methods

2.1. Rearing of Galleria mellonella

Larvae of the greater wax moth *Galleria mellonella* (Lepidoptera, Pyralidae) were reared at 27 ± 1 °C, 50–60% RH under darkness. An artificial medium was fed to the larvae—i.e. wheat flour 1000 g, corn flour 1000 g, wheat bran 600 g, Brewer's yeast 600 g, milk powder 600 g, bees wax 800 g, honey 1233 g, and glycerol 333 g (Shi et al., 2013). *G. mellonella* larvae (Sixth instars) were used during this study (Mylonakis, 2008).

2.2. Maintenance of Beauveria bassiana 70

The isolate *B. bassiana* 70 (*B. bassiana* sensu lato) was kindly provided by XinMin Li, Heilongjiang Academy of Agricultural Sciences, Heilongjiang Province, China. The original host of fungus is green peach aphid and geographical origin is Vladivostok, Russia (Khan et al., 2012). The fungus was maintained by culturing on potato dextrose agar (PDA) Petri plates (90 mm diameter) for 30 days at 26 °C in dark.

2.3. Preparation of fungal materials

2.3.1. Conidial suspension

Conidia were harvested from 20-day-old cultures on PDA (15 × 100 mm). Following the method of Khan et al. (2012), 2 mL of 0.02% tween 80 solutions were added to the culture and the spores gently removed from the mycelium using a glass spreader. The spore suspension was collected using a 2 mL syringe and placed in an Eppendorf tube (1.5 mL). The resulting suspension was then filtered through a sterile cheese cloth. The conidial concentration of the resulting suspension was estimated (1.0×10^8 conidia mL^{-1}) under a microscope using a haemocytometer. The viability of conidia, before use in bioassays, was confirmed using the method previously described by Hywell-Jones and Gillespie (1990).

2.3.2. Fungal filtrate

Based on the method of Quesada-Moraga et al. (2006a,b), the primary culture of the fungal isolate was prepared by adding 4 mL of conidial suspension (1.0×10^8 conidia mL^{-1}) into 100 mL of Adamek's liquid medium (Adamek, 1963). The culture was incubated in a rotary shaker at 26 °C with 200 rpm for three days. A secondary culture (1%) was prepared by adding 2.5 mL of primary culture into 250 mL of Adamek's liquid medium by incubating at 26 °C, 150 rpm for six days. The mycelia were separated by centrifugation for half an hour at 10,000 rpm at 4 °C, and the resulting supernatant was filtered through 0.45-μm-pore-size filter to obtain the filtrate.

2.3.3. Precipitation and concentration of crude proteins

Following Quesada-Moraga et al. (2006a,b), crude protein (CP) extract was precipitated with an 80% saturation rate of ammonium sulfate from culture filtrate of the fungal strain. The precipitate was then centrifuged at 10,000 rpm for 0 min at 4 °C— and the resultant pellets were dissolved in 40 mL of 20 mM tris–HCl buffer pH 7.4. Using dialysis tubing (SpectraPor molecular porous membrane) with a six to eight kDa cut-off membrane, the CP was desalted by dialyzing against 50 volumes of 20 mM tris–HCl pH 7.4 at 4 °C for 24 h. To concentrate the dialysates, polyethylene glycol (PEG) 20000 was used. A Bio-Rad Protein Assay kit was used to determine the total protein concentration, using Bovine serum albumin as a standard. Finally, a cocktail (Halt™ protease and Phosphatase Inhibitor Cocktail, Thermo Science) was mixed with the CPs and stored at −80 °C.

2.4. Identification, purification and characterization of the toxin protein

2.4.1. Electrophoresis

SDS–PAGE, with 12% acrylamide gel, was run for the CPs to find the molecular weight of proteins using Laemmli's (1970) method. The gel was destained for 8 h after staining for 4 h in 0.25% Coomassie Brilliant Blue-R dye, 40% methanol and 10% acetic acid. Prestained Protein Ladder (Fermentas #SM 0671) and SM1891 (Page Ruler™ Unstained Low Range Protein) were used as a marker.

2.4.2. Bioassay of crude protein extracts against G. mellonella

The CP was filter-sterilized through 0.22 μm Millex-GV device (Millipore Ireland BV, Carrigtwohill, Co Cork). A dose of 10 μl was injected into the *G. mellonella* larvae at concentrations of 0.952 mg/mL crude protein (0.4 mg/100 mg body weight). A 20 mM sterilized tris–HCl buffer pH 7.5 and BSA were injected as a control and a positive control, respectively. All the control and test *G. mellonella* larvae were incubated at 28 ± 1 °C in an incubator in Petri dishes. The whole procedure was repeated three times. The mortality was observed after 12 h for three days.

2.4.3. Protein purification

The crude protein was passed through a HiTrap Q HP column on an ÄKTA explore previously equilibrated with starting buffer (20 mM Tris–HCl, pH 7.5) at a flow rate of mL/min using 5 mL column. The unabsorbed fractions were collected and the absorbed fractions were collected in separate 50 mL tube using an elution buffer (Start buffer + 1 M NaCl). All the fractions purified were desalted and concentrated by ultra-filtration and were analyzed for activity against *G. mellonella*. Fractions having activity were further purified through Hydrophobic Interaction Chromatography (HIC) by passing (3 mL/min) the sample through a HiTrap phenyl FF (low sub) column (5 mL; GE Healthcare) previously equilibrated with the starting buffer (50 mM sodium phosphate + 1 M Ammonium sulfate). The unbound peaks were collected and an elution buffer (50 mM sodium phosphate) was used to elute the bound fraction of the proteins. The active portion of the protein was again purified through HiTrap phenyl FF (low sub) column with pH 7.0. The melanizing fraction of the protein was further applied on a

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