An inner cell wall protein (cwp1) from conidia of the entomopathogenic fungus *Beauveria bassiana*

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Following the removal of the rodlet layer from aerial or submerged conidia of the entomopathogenic deuteromycetous fungus *Beauveria bassiana*, SDS-insoluble, formic-acid-extractable proteins were found in the residual cell wall material. Two major proteins (12·8 and 14·0 kDa) were extracted with formic acid from fractured aerial and submerged conidia but not from blastospores. Oxidation of the sample extracted by formic acid resulted in a single protein band (15·4 kDa) as judged by SDS-PAGE. Antibodies against this cell wall protein (cwp1) did not cross-react with cell wall extracts from the entomopathogenic deuteromycetous fungi *Verticillium lecanii* or *Metarhizium anisopliae*. Western blot analysis of two-dimensional gels revealed at least three acidic isoforms (pI 4·0–4·8) of cwp1. Immunohistological studies revealed that the cwp1 was primarily localized in the cell wall of aerial and submerged conidia but not in blastospores. Immunolocalization was possible only if the conidia were previously boiled in 5% (v/v) β -mercaptoethanol. The N-terminal sequence of cwp1 showed no similarities with other published sequences. Our results suggest that at least two major species of SDS-insoluble, formic-acid-extractable proteins exist in cell walls of *B. bassiana* aerial or submerged conidia; one is the hydrophobin which occurs in the outermost rodlet layer and the other, cwp1, occurs primarily next to the rodlet layer.

Keywords: *Beauveria bassiana*, entomopathogenic fungus, conidia, cell wall protein

INTRODUCTION

Beauveria bassiana is an entomopathogenic fungus that is being considered as a commercial biocontrol agent. Several methods are available for propagation of *B. bassiana*. Aerial growth of hyphae on solid or semi-solid media results in the production of conidia, while submerged growth in nutrient-rich media results in the production of blastospores (Bidochka *et al.*, 1987). However, growth in certain types of liquid media, such as TKI medium (Thomas *et al.*, 1987), results in the production of 'submerged conidia'. Aerial and submerged conidia are similar with respect to their stability under storage, cell wall carbohydrates, hydrophobicity, virulence toward insects, and SDS-soluble proteins (Hegedus *et al.*, 1990, 1992). Aerial and submerged conidia also contain a rodlet layer. The major rodlet protein is SDS-insoluble and formic-acid-extractable and has been identified as a hydrophobin (Bidochka *et al.*, 1995). Hydrophobin was not found in blastospores.

Hydrophobins polymerize on the surface of conidia and this results in a water-impermeable, hydrophobic layer (de Vries *et al.*, 1993). This layer is, in part, responsible for protecting the conidium from desiccation (Cole *et al.*, 1979; Wessels, 1992). Conidial hydrophobicity also contributes to conidial dispersal (Bell-Peterson *et al.*, 1992; Lauter *et al.*, 1992) and, in the case of certain entomopathogenic fungi, to conidial attachment to the hydrophobic insect cuticle (Boucias *et al.*, 1988).

The ability of an entomopathogenic fungal conidium to resist desiccation, remain stable under long-term storage and to adhere to insect cuticle is an important consideration in its propagation as a biocontrol agent. We have previously identified a hydrophobin in the rodlet layer of *B. bassiana* conidia (Bidochka *et al.*, 1995). Here we investigate the presence of other SDS-insoluble, formic-acid-extractable proteins in cell walls of aerial and submerged conidia and blastospores of *B. bassiana*.

METHODS

Organisms and growth. The fungal isolates [*Beauveria bassiana* RS252, *Metarhizium anisopliae* 2575 (formerly ME1) and *Verticillium lecanii* 313] were obtained from the USDA entomopathogenic fungal culture collection in Ithaca, New York, USA, and maintained on Sabouraud dextrose agar at 25 °C to produce aerial conidia. Ten-day-old cultures containing aerial conidia were harvested in 0.02% Triton X-100, filtered through four layers of cheesecloth, and washed four times with water.

To obtain submerged conidia *B. bassiana* was grown in a modification of TKI medium (Thomas *et al.*, 1987) which consisted of (per litre distilled water): 50 g glucose, 10 g KNO₃, 5 g KH₂PO₄, 2 g MgSO₄, 50 mg CaCl₂·2H₂O, 50 mg yeast extract. Cultures were grown for 5 d at 25 °C and 180 r.p.m. Submerged conidia were obtained by filtering the culture through four layers of cheesecloth. The conidia were centrifuged at 3000 g and washed four times with water. To obtain blastospores, *B. bassiana* was propagated in 2% (w/v) peptone, 1% (w/v) glucose for 4 d at 25 °C and 180 r.p.m. (Bidochka *et al.*, 1987). The culture was filtered, and the blastospores washed four times with water.

B. bassiana was also grown in Reddy–Wyatt insect culture medium, which is a nutrient-rich medium analogous to insect haemolymph (Reddy & Wyatt, 1967), and in 1% (w/v) chitin medium.

Proteins were extracted from broth cultures by adding an equal volume of 0.1 M ammonium acetate in methanol to culture filtrates and left overnight at –20 °C. The precipitate was removed by centrifugation at 3000 g, washed with methanol and lyophilized.

Fracturing of conidial and blastospore cell walls. Rodlets were removed from *B. bassiana* by sonication as described by Boucias *et al.* (1988) except that the sonication time was extended another 5 min. The residual material was then centrifuged at 27000 g, washed with distilled water and lyophilized. Lyophilized conidia or blastospores (500 mg) and acid-washed sand (500 mg; 212–500 µm particle diameter) were then vortexed together with liquid nitrogen in a screwtop centrifuge tube (the cap was pierced to allow for expanding nitrogen) for three periods of 15 min each. The fractured fungal material and sand were then suspended in 20 ml 0.1 M Tris/HCl (pH 8.0). The sand was allowed to settle for 1 min and the supernatant containing the fractured cell walls was removed, centrifuged at 27000 g and washed twice each with Tris buffer and distilled water.

Dissolution of SDS-insoluble protein with formic acid. The fractured cell walls (post 27000 g centrifugation) were boiled in 100 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 10% (w/v) glycerol and 0.1% bromophenol blue for 10 min then washed six times (until the blue dye disappeared) with distilled deionized water and lyophilized. SDS-insoluble protein from the remaining cell wall material was extracted with trifluoroacetic acid or formic acid followed by oxidation with performic acid according to the method of Wessels *et al.* (1991).

PAGE and protein sequencing. Electrophoresis was done either in a 12% (w/v) polyacrylamide gel according to Laemmli (1970) or on a 4–20% gradient gel (Bio-Rad) and gels were silver-stained or stained with Coomassie blue. Bands of interest were excised from Coomassie-blue-stained gels, electroeluted according to manufacturer's guidelines (Bio-Rad), oxidized with performic acid, separated by SDS-PAGE and silver-stained.

For N-terminal protein sequencing, electrophoresis was done with the neutral buffer system described by Moos *et al.* (1988).

Gels were polymerized overnight and prerun at 10 mA per gel for 30 min. Electroblotting was done on PVDF membrane (Immobilin-P⁸⁰, Millipore) by tank transfer (Bio-Rad) at 100 V for 1 h. The membrane was stained in a fresh solution of Coomassie blue, destained for 2 h and bands of interest excised. The N-terminal sequence was determined by automated Edman degradation using an Applied Biosystems 47A gas-phase protein sequencer. Amino acid analysis was performed using the Waters (Waters Chromatography) Pico-Tag system (Henrikson & Meredith, 1984).

Two-dimensional gel electrophoresis was done according to manufacturer's guidelines (Bio-Rad).

Preparation of antibodies and immunoblotting. The SDS-insoluble, formic-acid-extractable proteins (with or without oxidation with performic acid) from fractured conidia cell walls were separated by SDS-PAGE. The major conidial wall protein from the performic-acid-treated sample was excised from SDS gels and homogenized in 250 µl distilled water. We called this protein cwp1. The suspension was sub-divided into three aliquots, mixed with Freund's complete adjuvant and injected 14 d apart into New Zealand White/Flemish-cross rabbits. Blood was taken after the final injection and the serum separated by centrifugation at 3000 g (4 °C).

For Western blot analysis, proteins were electrophoretically transferred from gels onto nitrocellulose. Blots were probed with anti-cwp1 (1:200 dilution) then visualized with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma). Western blot analysis was done according to the ProtoBlot Western blot alkaline phosphatase system technical manual (Promega) except that 1% (w/v) skimmed milk powder was used as a blocking agent.

Immunofluorescence microscopy. An indirect immunofluorescence technique was performed to localize the cwp1 antigens using primary and Cy3-labelled secondary antibodies. The secondary-antibody-Cy3 conjugate was affinity pure, goat anti-rabbit IgG (Sigma).

Conidia and blastospores were boiled in 5% (v/v) β-mercaptoethanol for 5 min. The material was centrifuged and washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄), pH 6.8. The material was resuspended in a 1:10 dilution of primary antibody (10 µl serum in 90 µl PBS). The suspension was incubated at 4 °C for 2 h with gentle agitation, washed three times with PBS at 4 °C, resuspended in a 1:10 dilution of secondary-antibody-Cy3 conjugate and incubated for 2 h at 4 °C with gentle agitation. After washing in PBS, a drop of the suspension was placed on a glass slide, examined immediately with a microscope and photographed with 1600 ASA film using a rhodamine filter. Control tests were performed with the omission of primary antibody.

Insect infection. *B. bassiana* (approximately 10⁶ conidia) was injected into the haemocoel of silkworm larvae, *Bombyx mori*, with a Hamilton repeating-volume syringe. Larvae died within 5 d and haemolymph was extracted 24 h and 48 h post-mortem, precipitated with an equal volume of 0.1 M ammonium acetate in methanol, washed with methanol and lyophilized.

RESULTS

SDS-insoluble, formic-acid-extractable conidial wall proteins

After removal of the rodlet layer by sonication, the conidia were fractured by vortexing with sand under liquid nitrogen. The SDS-insoluble wall proteins were

Fig. 1. SDS-insoluble proteins from fractured walls of aerial conidia, submerged conidia and blastospores of *B. bassiana*. Lanes: 1, formic acid extracts; 2, performic acid oxidation of the formic acid extracts; 3, performic acid extracts of the material remaining after formic acid extraction. Arrows indicate the two major proteins in the formic acid extract (lane 1: 12.8 and 14.0 kDa) and the major protein after performic acid oxidation (lane 2: 15.4 kDa). Proteins were subjected to 12.5% polyacrylamide gel electrophoresis followed by silver-staining. Molecular mass markers (kDa) are shown to the left of the gel.

extracted with formic acid from aerial conidia, submerged conidia and blastospores.

Two major protein bands (12.8 kDa and 14.0 kDa) were observed in the formic acid extracts of fractured aerial and submerged conidia but not in blastospores (Fig. 1, lane 1). Oxidation of the formic acid extracts with performic acid resulted in a single major protein of larger molecular mass (15.4 kDa; Fig. 1, lane 2) termed cwp1. Performic acid treatment of the residual fungal cell wall material after formic acid treatment (Fig. 1, lane 3) showed results similar to oxidation of the formic acid extract. Tri-fluoroacetic acid extracts of the wall proteins also resulted in a single 15.4 kDa protein (data not shown). The 12.8 kDa and 14.0 kDa bands were excised separately from gels and oxidized with performic acid. Both of the oxidized proteins ran as a single band of 15.4 kDa on SDS-PAGE (data not shown).

Immunological detection of cwp1

Antiserum to cwp1 from aerial conidia showed immuno-reactivity to aerial and submerged conidia wall proteins extracted with formic acid and oxidized with performic acid (Fig. 2, lane 2). However, without the performic acid treatment, formic acid wall extracts did not cross-react with cwp1 antibodies (Fig. 2, lane 1). cwp1 was not detected in blastospore extracts.

Western blot analysis of precipitated proteins (with or without oxidation with performic acid) from *B. bassiana* culture filtrates of Reddy-Wyatt insect culture medium, 1% (w/v) chitin medium, 2% peptone-1% (w/v) glucose or haemolymph from infected silkworm larvae did not detect the presence of cwp1 at the 15.4 kDa position. However, the cwp1 antibody did cross-react with a 28 and

Fig. 2. Immunoblot from a 4–20% polyacrylamide gradient gel using antiserum against cwp1 of SDS-insoluble proteins from fractured walls of aerial conidia, submerged conidia and blastospores of *B. bassiana*. Lanes: 1, formic acid extracts; 2, performic acid oxidation of formic acid extracts. Molecular mass markers (kDa) are shown to the left of the gel.

32 kDa doublet from *B. bassiana* culture filtrates in Reddy-Wyatt medium and 2% peptone, 1% glucose medium in both the oxidized and unoxidized samples (data not shown).

Fig. 3. SDS-insoluble proteins from fractured walls of aerial conidia were treated with performic acid and subjected to two-dimensional polyacrylamide gel electrophoresis then immunoblotted using anti-serum against cwp1. Arrows indicate the three isoforms of cwp1. Isoelectric markers (pI; top) and molecular mass markers (kDa; left) are shown.

Fig. 4. Bright field (top) and immunofluorescence (bottom) micrographs of (a) submerged conidia developing from hyphae, (b) microcycle conidiation of submerged conidia, (c) aerial conidia and (d) blastospores of *B. bassiana*. Samples were boiled in 5% β -mercaptoethanol and subjected to immunofluorescence labelling with antiserum against cwp1. Bar, 10 μ m.

Immunoblotting of the oxidized (performic-acid treated) protein after two-dimensional gel electrophoresis showed that the cwp1 has at least three isoforms ranging from pI 4.0 to 4.8 (Fig. 3).

The cwp1 antibodies showed no immunoreactivity to the SDS-insoluble, formic acid/performic-acid-treated proteins from conidia of the entomopathogenic fungi *M. anisopliae* or *V. lecanii*.

Immunofluorescence of cwp1 *in vivo*

Fig. 4(a) shows the formation of submerged conidia from hyphal structures after 3 d growth in modified TKI broth. Mature conidia fluoresced more strongly than either the immature conidia or the hyphae. Microcycle conidiation was observed in TKI broth (Fig. 4b). The germinated conidia, the stalk and newly formed conidia fluoresced. Aerial conidia boiled with β -mercaptoethanol then treated

Table 1. Amino acid analysis of *B. bassiana* cwp1

Residue	nmol	nmol ratio*	No. of residues
Ala	0.601	1.00	12
Arg	0.207	0.34	4
Asx	0.649	1.08	13
Cys	0.313	0.52	6
Glx	0.527	0.88	10
Gly	1.339	2.23	28
His	0.247	0.41	5
Ile	0.292	0.49	6
Leu	0.882	1.47	17
Lys	0.341	0.56	8
Met	0.073	0.12	1†
Phe	0.057	0.09	1†
Pro	0.277	0.46	6
Ser	0.548	0.91	11
Thr	0.531	0.88	11
Tyr	0.163	0.27	3
Val	0.432	0.72	9

* Ratio of nmol to those of Ala.

† To obtain a molecular mass value approximated to that calculated by SDS-PAGE we assumed one residue each of Met and Phe per molecule. Assuming the calculated amino acid residue number, the molecular mass is 16079 Da.

with cwp1 antibody fluoresced with Cy3 (Fig. 4c). Blastospores showed very weak or no fluorescence (Fig. 4d). Aerial or submerged conidia fluoresced weakly if not previously boiled in β -mercaptoethanol. Aerial conidia fluoresced more strongly if previously sonicated to remove cell wall surface rodlets (data not shown).

Amino acid analysis

Sequencing of the N-terminus of cwp1 revealed the sequence: Ser-Gly-His-His-Gly-Gly-Lys-Asp-Leu-His-Lys-X'-Thr-Leu-Gly-Glu-Ala-Gly-Asp-Arg-X-Gly-Ala-Asp (where X' is a non-standard amino acid or a modified amino acid but not hydroxyproline and X is an unresolved amino acid). Sequence analyses were identical for cell wall protein extracts from aerial or submerged conidia. A search for homologous sequences (GCG gene analysis package available to us as a service from the Cornell Biotechnology Program) showed no significant relationships between cwp1 and other fungal proteins.

Amino acid analysis of cwp1 is presented in Table 1. The protein is rich in glycine (18.5% of residues) and leucine (11% of residues). The molecular mass estimated by amino acid analysis is 16079 Da, which is in close approximation to the molecular mass estimated by SDS-PAGE (15400 Da). cwp1 has a polarity index (Capaldi & Vanderkooi, 1972) of 42.3, indicating that in total it is a slightly hydrophobic protein in spite of the N-terminus containing an abundance of hydrophilic amino acids such as histidine, lysine, aspartic acid, glutamic acid, threonine and arginine. However, amino acid analyses should be

interpreted cautiously since the reliability differs for different amino acid residues and cysteine residues are particularly difficult to assess.

DISCUSSION

After removal of the rodlet layer from *B. bassiana* conidia, SDS-insoluble, formic-acid-extractable proteins with molecular masses of 12.8 kDa and 14.0 kDa were found in fractured cell walls of aerial conidia and submerged conidia. Performic-acid treatment of this material resulted in a shift toward a lower mobility in the SDS-PAGE to an apparent single product of 15.4 kDa (cwp1). Oxidation of hydrophobins extracted from rodlets of *B. bassiana* (Bidochka *et al.*, 1995) and from cell walls of other fungi (de Vries *et al.*, 1993) also resulted in a shift toward a lower mobility. Oxidation of cysteine residues to cysteic acids results in relaxation of the protein so that the apparent molecular mass increased. We had initially believed cwp1 to be of the 'hydrophobin' class of fungal wall proteins since it filled several of the criteria in being (1) a low-molecular-mass protein from fungal walls which was (2) SDS-insoluble, (3) soluble in formic acid, and (4) when oxidized with performic acid the resultant protein was of higher molecular mass. cwp1 contains at least six cysteine residues which could be involved in disulfide bond formation. N-terminal sequence analysis revealed no homologies to known hydrophobins including the *B. bassiana* rodlet protein (Bidochka *et al.*, 1995). However, this does not necessarily exclude cwp1 as a hydrophobin. The N-terminal region of the RodA hydrophobin from *Aspergillus nidulans* (Stringer *et al.*, 1992) does not show sequence similarities with other hydrophobins (Stringer & Timberlake, 1993). The question of whether cwp1 is a hydrophobin can only be settled by internal amino acid sequence analysis or from the coding sequence of cwp1 cDNA.

Hydrophobin and cwp1 are apparently important to the insoluble complex of the walls of aerial and submerged conidia but not blastospores of *B. bassiana*. It is not uncommon for more than one SDS-insoluble protein complex to be found in walls of fungi. Three hydrophobin-like proteins are found in *Schizophyllum commune* (Wessels *et al.*, 1991) and one, two or three SDS-insoluble, formic-acid-soluble proteins were found in Basidiomycetes and Ascomycetes (de Vries *et al.*, 1993). cwp1 may be specific to *B. bassiana* since *M. anisopliae* and *V. lecanii* cell wall proteins did not immunoreact with the cwp1 antibody.

B. bassiana cwp1 and hydrophobin may represent the major SDS-insoluble proteins of the inner and outer cells walls in conidia, respectively. An electron microscopic study of conidiogenesis and wall formation in *Aspergillus niger* revealed that the inner and outer cell walls of mature conidia are laid down by the conidium protoplast (Tiedt, 1993). The electron-dense surface coating originates from secreted material which polymerizes when exposed to air. This is consistent with the observation of Wessels (1992) that hydrophobins polymerize on the fungal cell wall when exposed to air. The electron-dense surface coating is

also consistent with the outer rodlet layer, composed primarily of hydrophobin (Bidochka *et al.*, 1995; Bell-Peterson *et al.*, 1992; Stringer *et al.*, 1992; Lauter *et al.*, 1992). Removal of the outer rodlet layer revealed that cwp1 is the major SDS-insoluble protein underlying the rodlet layer in *B. bassiana*.

B. bassiana cwp1 and hydrophobin are both found in aerial or submerged conidia but not in blastospores and may be a class of developmentally regulated proteins that appear to be produced concurrently. We can only speculate as to the reasons why cwp1 is found in submerged conidia and not in blastospores. cwp1 may be regulated through environmental cues through differences in the culture medium as previously outlined for hydrophobin production in *B. bassiana* (Bidochka *et al.*, 1995).

When fungi are grown under submerged conditions hydrophobins are released into the extracellular medium (Wessels, 1992). This is not clearly so for cwp1. The cwp1 antigen did not immunoreact with a 15.4 kDa protein from culture filtrates or proteins extracted from infected insect haemolymph. The antibody did, however, react with higher molecular mass proteins and this could represent cwp1 complexes, or a nonspecific interaction.

Strategies for propagating *B. bassiana* and other entomopathogenic fungi may influence cell wall properties of the fungus which affect its viability, hydrophobicity and ability to infect insects. Further work examining the regulation, production, deposition and assembly of cwp1 and hydrophobin on the inner and outer cell walls is required.

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