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Identification and partial characterization of an elastolytic protease in the amphibian pathogen *Batrachochytrium dendrobatidis*

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ABSTRACT: *Batrachochytrium dendrobatidis* (*Bd*) is a fungus that causes chytridiomycosis, a disease that has been implicated as a cause of amphibian population declines worldwide. Infected animals experience hyperkeratosis and sloughing of the epidermis due to penetration of the keratinized tissues by the fungus. These symptoms have led us to postulate that *Bd* produces proteases that play a role in the infection process. Here, we show that *Bd* is capable of degrading elastin *in vitro*, a protein found in the extracellular matrix of the host animal. Elastolytic enzyme activity was partially purified using ion exchange chromatography and size-exclusion filtration from cultures grown in inducing media. The elastolytic activity of the purified fraction had a pH optimum of 8, was strongly inhibited by EDTA and phenylmethylsulfonyl fluoride (PMSF), and was partially inhibited by an elastase-specific inhibitor. This activity was also enhanced by the presence of Mg^{2+} and Ca^{2+} but not Zn^{2+}. An antiserum directed against *Aspergillus fumigatus* serine protease (Alp) was found to react with a polypeptide of approximately 110 kDa from the purified material. Using immunofluorescence, this antiserum was also observed to react with zoospores and sporangia grown on toad skin. These observations suggest that *Bd* may produce proteases similar to those produced by other pathogenic fungi that are capable of degrading proteins found in the extracellular matrix. The proteolytic activity exhibited *in vitro* might aid the organism in its ability to colonize and destroy the epidermis of its amphibian host.

KEY WORDS: Chytridiomycosis · Pathogenicity · Amphibians

INTRODUCTION

Batrachochytrium dendrobatidis (*Bd*) causes chytridiomycosis, a serious disease of amphibians now believed to be responsible for widespread global declines in amphibian populations (Daszak et al. 1999, Longcore et al. 1999, Wake & Vredenburg 2008, Woodhams et al. 2008). The organism produces wall-less zoospores that are waterborne and motile by means of a single, posterior flagellum. Zoospores swim through their aquatic environment until they find a suitable substrate upon which to encyst. They then absorb their flagellum, form a cell wall, and develop into a zoosporangium (Berger et al. 2005). Chytridiomycosis is characterized by hyperkeratosis and colonization of the keratinized layers of the amphibian epidermis by chytrid sporangia (Bradley et al. 2002). The details of the pathogenicity of this organism are not completely understood.

Certain microorganisms possess the ability to secrete proteolytic enzymes into their environment. Some organisms do this in an effort to hydrolyze large polypeptides into smaller substrates for metabolism (Wolf & Ehmann 1981, Apodaca & McKerrow 1989), while others use them as a means to break down protein barriers in their host (Dickman et al. 1982, Kolattukudy et al. 1993, Cascon et al. 2000). These capabilities are more commonly seen in pathogenic forms of

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microorganisms such as *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, and *Trichophyton* sp. that colonize areas such as the skin, lungs, and eyes (Howe & Iglewski 1984, Kothary et al. 1984, Hung et al. 2005, Kaufman et al. 2005).

Batrachochytrium dendrobatidis produces extracellular proteases that are effective in the degradation of trypsin and chymotrypsin (Symonds et al. 2008), skim milk and gelatin, but not keratin (Piotrowski et al. 2004). Additionally, the recent sequencing of the genomes of 2 isolates of the organism by the Broad Institute (www.broad.mit.edu) and the U.S. DOE Joint Genome Institute (www.jgi.doe.gov) revealed the presence of an extracellular elastinolytic metalloproteinase. An expansion in the *Bd* genome of 25 genes containing a peptidase M36 domain was reported by Rosenblum et al. (2008). This domain is seen in fungalysin metalloproteases, enzymes commonly seen in dermatophytic fungi (Brouta et al. 2002, Monod et al. 2002). In addition to the M36 family, an expansion of the serine-type peptidases is seen in this organism. These enzymes are also proposed to play a role in host protein degradation (Resnick et al. 1987, Apodaca & McKerrow 1989, Reichard et al. 1990, Monod et al. 1991, Jaton-Ogay et al. 1992, Kolattukudy et al. 1993, Ramesh et al. 1994, Rosenblum et al. 2008).

The above information, along with the ability of *Batrachochytrium dendrobatidis* to colonize and proliferate in the skin of its amphibian host, led us to investigate the ability of *Bd* to produce proteolytic enzymes specific to skin-related substrates by this organism.

MATERIALS AND METHODS

Fungal culture conditions. *Batrachochytrium dendrobatidis* (isolate VM1) was provided by Louise Rollins-Smith (Vanderbilt University). This isolate was obtained from a diseased western chorus frog *Pseudacris triseriata* by Verma Miera and Elizabeth Davidson of Arizona State University. The isolate was cultured and maintained on TGhL agar plates (1.6% tryptone, 0.2% gelatin hydrolysate, 0.4% lactose, and 0.8% agar) or H-broth (1% tryptone, 0.32% glucose) according to methods outlined by Boyle et al. (2003). VM1 was also grown in 1% non-fat dry milk (1% NFDM) (1% NFDM, 10 mM Tris, pH 7.5). Culture supernatants from H-broth and 1% NFDM were assayed for elastase activity.

Toad skin inoculations. To study *Batrachochytrium dendrobatidis* infections on skin *in vitro*, zoospores were inoculated onto surface-sterilized toad skin harvested from healthy New Mexico spadefoot toads *Spea multiplicata* that were collected from playa lakes in the Lubbock, Texas, area. Each piece of skin used in the experiment was sterilized by immersion in 95% ethanol with subsequent rinses in sterile distilled water to remove the residual ethanol. A single piece of sterilized skin was placed onto the center of each of 10 TGhL agar plates. Zoospores were harvested from TGhL agar plates (5 to 7 d old) with dilute salts (DS) water (2×10^{-5} M $CaCl_2$, 10^{-4} M $MgCl_2$, 10^{-3} M KH_2PO_4) using routine methods (Boyle et al. 2004). A 0.1 ml volume suspension containing approximately 2×10^6 zoospores was inoculated onto each of 5 pieces of toad skin. The other 5 pieces of skin were sham inoculated with DS water as negative controls. All 10 plates were wrapped in Parafilm™, incubated at 25°C, and monitored over a period of 5 d. Starting with Day 1 and continuing with each day thereafter, 2 pieces of skin (one from the inoculated group and one from the uninoculated group) were removed from the TGhL agar plate, fixed in 2% glutaraldehyde (Ted Pella) and 4% osmium tetroxide (Ted Pella) and passaged through a stepwise dehydration series before being embedded in Spurr's epoxy resin (Ted Pella). Thick sections (approximately 0.5 to 2.0 µm in thickness) were cut and stained with 1% toluidine blue before being visualized with an Olympus BH-2 light microscope fitted with an Olympus DP70 digital camera (Olympus).

Characterization of crude culture supernatant. *Batrachochytrium dendrobatidis* cultures (5 d old) grown in 1% NFDM were tested for enzymatic activity.

Assays for enzymatic activity: The protein elastin, found in the extracellular matrix of animals, plays an important role in tissue structure. Amphibians infected with *Batrachochytrium dendrobatidis* experience hyperkeratosis and sloughing of the epidermis. Therefore, we investigated the ability of *Bd* to degrade the elastin component of amphibian skin. Elastolytic activity was measured using DQ™ elastin (Invitrogen) as a substrate. DQ™ elastin is a soluble bovine neck ligament elastin that has been labeled with a fluorescent dye (BODIPY® FL). Reactions were carried out using 96-well Lumitrac™ 600 white immunology plates (Grenier Bio-One) that were monitored with an FL600 fluorescent plate reader (Biotek Instruments). Reaction well contents included the enzyme solution (supernatant fractions harvested from *Bd* grown in 1% NFDM) and 5 µg of DQ™ elastin in 100 mM Tris-HCl, pH 8.0. Brefeldin A (Sigma) and monensin (Sigma), inhibitors that interfere with intracellular protein transport, were added to *Bd* cultures 24 h prior to being assayed for elastolytic activity. Negative controls included 1% NFDM in which *Bd* was not cultured.

Growth pH optimum for elastase production: The optimum pH for *Batrachochytrium dendrobatidis* growth is between 6 and 7 (Piotrowski et al. 2004). The relative acidity or alkalinity of a medium can influence growth and production of enzymes. We therefore eval-

uated the range of pH in which enzyme production in *Bd* was maximized. The effect of pH on induction of the elastolytic enzyme was determined by culturing *Bd* in 1% NFDM in buffers of varying pH. The buffers used included 10 mM m2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5); 10 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.5); and 10 mM Tris (pH 7.5, pH 8.5, and pH 9.5). Cultures were incubated at 25°C for 5 d before being assayed for elastase activity.

Electrophoresis: Equal amounts of protein preparations were analyzed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970). Novex® Tris-glycine pre-cast gels (Invitrogen) were also used. Gels were stained either with Coomassie Brilliant Blue R-250 (Amresco) or silver nitrate (Fisher).

Partial purification of enzyme from crude supernatant. The crude culture supernatants contained a large number of proteins. For this reason, the supernatants were fractionated on the basis of size, and the proteins responsible for elastolytic activity were analyzed further. Seventy-milliliter batches of culture supernatants of *Batrachochytrium dendrobatidis* (grown in 1% NFDM) were concentrated using Centricon Plus-70 centrifugal filter units (Millipore) with a molecular weight cut-off of 30 kDa. The filtrate (containing proteins smaller than 30 kDa) and the retentate (containing proteins 30 kDa and larger) were tested for elastolytic activity using DQ elastin™ as a substrate. The retentate showed the largest amount of elastolytic activity and was subsequently transferred to a diethylaminoethyl-cellulose (DEAE-cellulose) anion exchange column that was washed and equilibrated with 30 mM Tris buffer (pH 8.0) containing 50 mM NaCl. Fractions were eluted with 30 mM Tris (pH 8.0) containing 100 mM NaCl, 150 mM NaCl, and 200 mM NaCl, respectively. Each fraction was lyophilized before being dialyzed against a stepwise series of buffers containing 1M NaCl, 500 mM NaCl, 250 mM NaCl, and finally 10 mM Tris buffer (pH 8.0).

Characterization of the partially purified enzyme. The partially purified material was tested for elastolytic activity, and the effect of metal ions, pH, and inhibitors was evaluated as described below, using DQ elastin™ as a substrate. This material was also subjected to zymography to determine the size and presence of proteolytic proteins.

Metal ion optimum for elastase activity: Some proteins require binding of a divalent metal ion in their catalytic site for activity and may also be classified accordingly (Rao et al. 1998). Divalent metal cations ($MgSO_4$, $CaCl_2$, $ZnSO_4$) were added separately to the assay at a final concentration of 1 mM in order to examine the effect of each on elastolytic activity.

pH optimum for elastase activity: Enzymes display pH optima at which their activity is maximal. The catalytic activity of enzymes may be regulated by the pH of the surrounding environment. The same buffers used in the enzyme production experiments above were used in the elastase assay in order to determine the optimal pH for elastase activity.

Effect of inhibitors on elastase activity: Proteases can be classified according to their sensitivity to various inhibitors. We used a metal ion chelator (EDTA); a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF); and an elastase-specific inhibitor (*N*-methoxysuccinyl-ala-ala-pro-val-chloromethyl ketone). Inhibitors were pre-incubated with the enzyme solution and reaction buffer for 30 min before the addition of the DQ™ elastin substrate.

Data analysis: The bars on all enzymatic assays represent standard error of the mean. Significance was assessed using a Student's *t*-test.

Protein estimation: Total protein concentrations for enzyme samples were determined according to the Bradford method (Bradford 1976) using Bio-Rad Protein Assay Dye (Bio-Rad).

Zymography: Zymograms impregnated with 0.1% gelatin (10% Tris-glycine) or 0.05% casein (12% Tris-glycine) (Invitrogen) were run in an effort to determine the presence and molecular sizes of proteases that utilize gelatin or casein in the material. Equal amounts of proteins were separated on zymograms under denaturing conditions and then renatured and developed using Novex® Zymogram Renaturing Buffer and Novex® Zymogram Developing buffers (Invitrogen), respectively. After developing overnight at 37°C, the zymograms were stained using a Colloidal Blue Staining kit (Invitrogen).

Immunoblot analysis: Immunoblots were performed using the XCell SureLock™ Mini Cell electrophoresis system (Invitrogen) along with the XCell II™ Blot Module (Invitrogen). Equal amounts of proteins were separated using SDS-PAGE before being transferred onto polyvinylidene fluoride (PVDF) (Millipore) membranes and blocked with 5% NFDM. Membranes were then probed with the *Aspergillus fumigatus* serine protease antiserum, followed by an alkaline phosphatase conjugated secondary antibody (Southern Biotech). Membrane sites bearing alkaline phosphatase were developed with Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega). Rabbit pre-immune serum (Rockland Immunochemicals) was used as a control for the primary antiserum.

Immunodetection of proteolytic enzyme. Zoospores of *Batrachochytrium dendrobatidis* were inoculated onto surface sterilized toad skin (as described above) harvested from healthy New Mexico spade-

foot toads *Spea multiplicata*. A single piece of sterilized skin was placed onto the center of each of 2 TGhL agar plates. Zoospores were harvested from TGhL agar plates (5 to 7 d old) as outlined above, and a 0.1 ml volume suspension containing approximately 2×10^6 zoospores was inoculated onto one piece of toad skin (Treatment 1). A sham inoculum of DS water was placed onto the other piece of skin (Treatment 2). Another 0.1 ml suspension of zoospores was placed onto the surface of a TGhL agar plate containing no toad skin (Treatment 3). The plates were wrapped in Parafilm™ and allowed to incubate at 25°C for 5 d. Zoospores were floated from Treatments 1 and 3, concentrated by centrifugation, and the resulting pellets containing zoospores were re-suspended in blocking buffer (10 mM potassium phosphate, 2% fetal bovine serum, pH 7.6). The TGhL agar plate containing uninoculated toad skin was floated in the same manner as above, and the resulting liquid suspension (containing no zoospores) was centrifuged and re-suspended in blocking buffer. All 3 treatments were placed in a chamber slide (Nalge Nunc) treated with poly-L-lysine (Ted Pella), and incubated at 4°C for 24 h. The treatments were then incubated at 4°C, overnight, with *Aspergillus fumigatus* serine protease antiserum (Linda Rogers, University of Central Florida) diluted 1:500 with blocking buffer. After incubation with the primary antiserum, the slide was washed with blocking buffer before being incubated with the rhodamine conjugated secondary antibody (Chemicon) (diluted 1:500 in blocking buffer) for approximately 2 h at 25°C. The slide was then viewed with an Olympus BX50 epifluorescence microscope (Olympus). Rabbit pre-immune serum (Rockland Immunochemicals) was used as a negative control for the primary antiserum.

RESULTS

Effect of *Batrachochytrium dendrobatidis* on toad skin

Batrachochytrium dendrobatidis is recognized as a pathogen that infects the epidermis of amphibians. We therefore investigated the ability of the fungus to proliferate on epidermal tissue from animals collected locally. Toad skin inoculated with *Bd* zoospores showed extensive degradation following 5 d of exposure to the fungus at room temperature. Histological examination showed disorganization of the epidermal layers in toad skin exposed to *Bd*, while unexposed skin was not affected (Fig. 1). These results suggested that *Bd* produces proteins necessary for the breakdown of amphibian tissue.

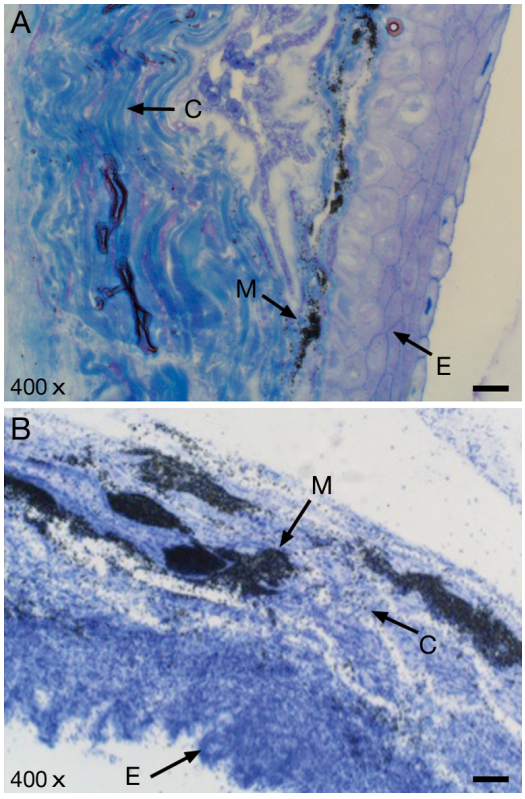

Fig. 1. *Spea multiplicata*. Thick sections of toad skin after 5 d exposure to (A) dilute salts (DS) water and (B) *Batrachochytrium dendrobatidis*. Arrows indicate epidermis (E), collagen (C), and melanin (M) components. Scale bars = 20 µm. Complete tissue degradation *in vitro* may explain the absence of sporangia which were likely lost during the fixation step. The apparent increase in melanization is probably due to dispersal of melanin when the skin was degraded. Figure is representative of multiple observations

Assays for enzymatic activity

Our observation of amphibian tissue degradation *in vitro* led us to hypothesize that proteins present in the amphibian epithelium, such as elastin, collagen, and keratin, may be the target of *Batrachochytrium dendrobatidis* enzymes. Of these 3 substrates, we observed notable elastase degradation *in vitro.* The greatest amount of elastase activity was seen when *Bd* was grown in 1% NFDM over a period of 5 d as compared with H-broth (data not shown). The choice of 1% NFDM was based on previous observations that *Bd* can degrade casein (Piotrowski et al. 2004) and is attracted to casein (Moss et al. 2008). Therefore, 5-d-old cultures of *Bd* grown in 1% NFDM were used for all elastase characterization studies. Crude culture supernatants of *Bd* grown in this medium showed a dramatic amount of elastolytic activity when compared to the uninoculated control. Additionally, cultures grown at a pH of 7.5 yielded the highest amount of proteolytic activity.

Effect of secretory inhibitors on elastase activity

In order to define the mechanism underlying secretion of elastolytic activity, specific inhibitors were used in *Batrachochytrium dendrobatidis* culture media. Brefeldin A and monensin were added to partially grown cultures 1 d prior to being assayed. Brefeldin A, at a concentration of 10 µM, resulted in a 26% decrease in elastolytic activity compared with control cultures. Monensin, at an equal concentration, resulted in a 15% decrease in elastolytic activity.

Partial purification of elastolytic enzyme

SDS-PAGE of the crude culture supernatant fractions of *Batrachochytrium dendrobatidis* grown in 1% NFDM revealed the presence of a complex mixture of polypeptides. We used size fractionation and ion exchange chromatography in an attempt to isolate the enzymes responsible for elastolytic activity. The elastolytic enzyme was purified 11-fold (Table 1), with the highest amount of specific activity (approximately 90%) seen in the 150 mM NaCl eluted fraction (Fig. 2). The 100 mM and 200 mM NaCl eluates showed minimal activity (data not shown). The pH optimum for elastolytic activity with this fraction was 8, compared to the crude supernatant, which was between 6.5 and 7.5 (data not shown). Six protein bands remained in this fraction (visualized with silver stain) (Fig. 3). Of these 6, a large band (approximately 110 kDa) showed proteolytic activity on gelatin- and casein-impregnated gels (Fig. 4). The smaller (approximately 46 kDa) band in Lane 5 showing proteolytic activity may be a degradation product of the larger 110 kDa polypeptide, or another co-purifying protein from the column.

Table 1. Partial purification of the elastolytic enzyme activity of *Batrachochytrium dendrobatidis* from the culture supernatant using size exclusion and ion exchange chromatography. Total act.: total activity; sp. act.: specific activity

Step	Vol. (ml)	Total protein (µg)	Total act. (U)	Yield (%)	Sp. act. (U µg^{-1})	Purification (fold)
Supernatant	70	9000	700	100	0.07	1.0
Size exclusion	10	8000	200	29	0.03	0.42
Ion exchange	3	75	60	8.6	0.8	11.4

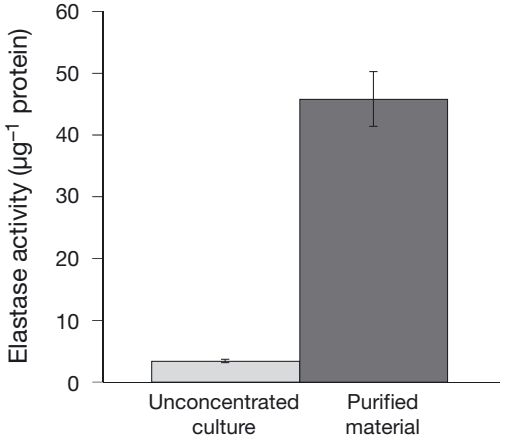

Fig. 2. Partial purification of *Batrachochytrium dendrobatidis* culture supernatants (grown in 1% non-fat dry milk [NFDM], pH 7.5) using ion exchange chromatography. The purified material yielded an 11-fold increase in elastolytic activity when compared to crude culture supernatants. Bars indicate standard error of the mean. A Student's *t*-test indicated a significant difference between the amount of elastolytic activity seen with the crude culture supernatant and the purified culture supernatant ($p < 0.001$)

Effect of inhibitors and metal ions on elastase activity seen with partially purified material

The effect of inhibitors on elastase activity was examined by incubating the appropriate inhibitory

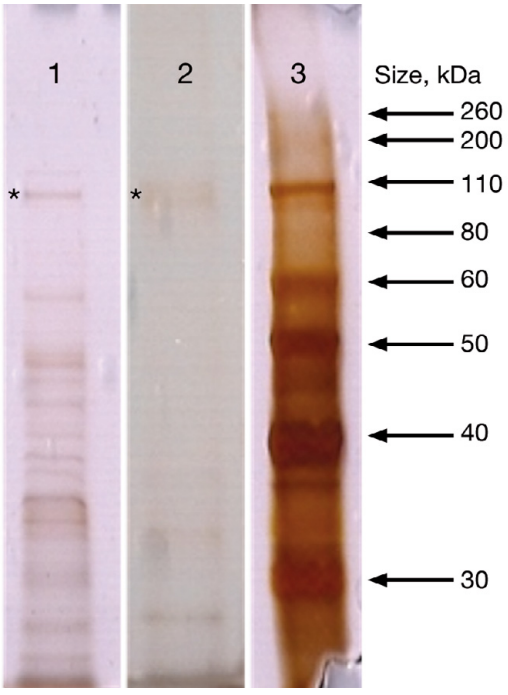

Fig. 3. 8% Tris-glycine gel (silver stained) of culture supernatant and purified culture supernatant (Lanes 1 and 2, respectively) of *Batrachochytrium dendrobatidis* grown in 1% non-fat dry milk. Asterisks indicate bands at >100 kDa. These bands appear to be proteolytic when separated on Tris-glycine gels impregnated with 0.1% gelatin and 0.05% casein (zymograms). Lane 3 contains molecular weight standard

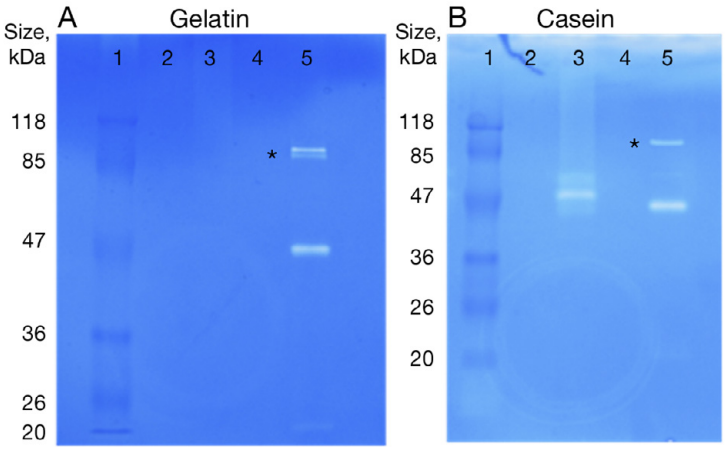

Fig. 4. 10% and 12% Tris-glycine protein gels impregnated with (A) 0.1% gelatin and (B) 0.05% casein, respectively, were used to demonstrate proteolytic activity in the purified material. (A) Asterisk in Lane 5 indicates a proteolytic band at approximately 110 kDa. (B) Asterisk in Lane 5 indicates a proteolytic band at approximately 110 kDa. Lanes 3 and 1 in both gels contain unpurified culture supernatants and molecular weight standard, respectively. The smaller (approximately 46 kDa) band in Lane 5 showing proteolytic activity may be a degradation product of the larger 110 kDa polypeptide, or another co-purifying protein from the column

agent with the enzyme solution and all other reaction contents excluding the substrate for a period of 30 min before the assay (Table 2). Inhibitors tested included *N*-methoxysuccinyl-ala-ala-pro-val-chloromethyl ketone (Invitrogen) (elastase-specific inhibitor), EDTA (metal ion chelator), and PMSF (serine protease inhibitor). The divalent cation chelator, EDTA, and serine protease inhibitor, PMSF (at concentrations of 1 mM each), had the greatest effect on elastolytic activity seen with the purified material, reducing this activity to 24 and 32%, respectively (Table 2). The elastase-specific inhibitor (*N*-methoxysuccinyl-ala-ala-pro-val-chloromethyl ketone), at a concentration of 0.5 mM, reduced activity by 23%. The solvent controls for the elastase-specific inhibitor (DMSO) and PMSF (isopropanol) did not have an inhibitory effect on elastolytic activity. The addition of 1 mM Ca^{2+} resulted in a dramatic 65% increase in elastolytic activity. Mg^{2+} (1 mM) increased enzyme activity by 23%. Zn^{2+}, at a concentration of 1 mM, had an inhibitory effect on enzyme activity, reducing it by approximately 40% (Table 3).

Table 2. Inhibition of purified elastolytic protease activity. PMSF: phenylmethylsulfonyl fluoride; *N*-MAAPV: *N*-methoxysuccinyl-ala-ala-pro-val-chloromethyl ketone

Inhibitor	Concentration	Activity remaining (%)
No inhibitor		100
EDTA	1 mM	24
PMSF	1 mM	32
N-MAAPV	0.5 mM	77
Isopropanol[a]	99.5%	110

[a]Isopropanol was used as a solvent control

Immunoblot analysis

Using the purified material, we observed cross-reactivity with a high molecular weight polypeptide of approximately 110 kDa in immunoblots (data not shown). No reactivity was observed with the pre-immune antibody.

Immunofluorescence

Indirect immunofluorescence studies revealed that *Batrachochytrium dendrobatidis* zoospores and sporangia, exposed to toad skin over a period of 5 d, displayed immunoreactivity with an antiserum directed against *Aspergillus fumigatus* serine protease (Fig. 5). Zoospores and sporangia that were not exposed to skin also showed fluorescence. Rabbit pre-immune serum, used as a control, did not exhibit fluorescence, nor did zoospores that were not treated with the rhodamine-conjugated secondary antibody.

DISCUSSION

The observations in this paper support the hypothesis that *Batrachochytrium dendrobatidis* produces proteolytic enzymes capable of degrading elastin, as well as gelatin and casein, *in vitro*. *Bd* is known to cause hyperkeratosis and sloughing of the amphibian epidermis in susceptible animals. Therefore, we inoculated zoospores onto the surface of sterilized toad skin in an effort to investigate this interaction. After 5 d, extensive degradation and disorganization of the epidermal layers were evident in inoculated skin, while uninoculated toad skin remained intact. These results suggest that the zoospores secreted an enzyme or combination

Table 3. Effect of metal ions on elastolytic activity

Metal ion	Concentration	Activity (%)
No metal		100
Mg^{2+}	1 mM	123
Ca^{2+}	1 mM	165
Zn^{2+}	1 mM	62

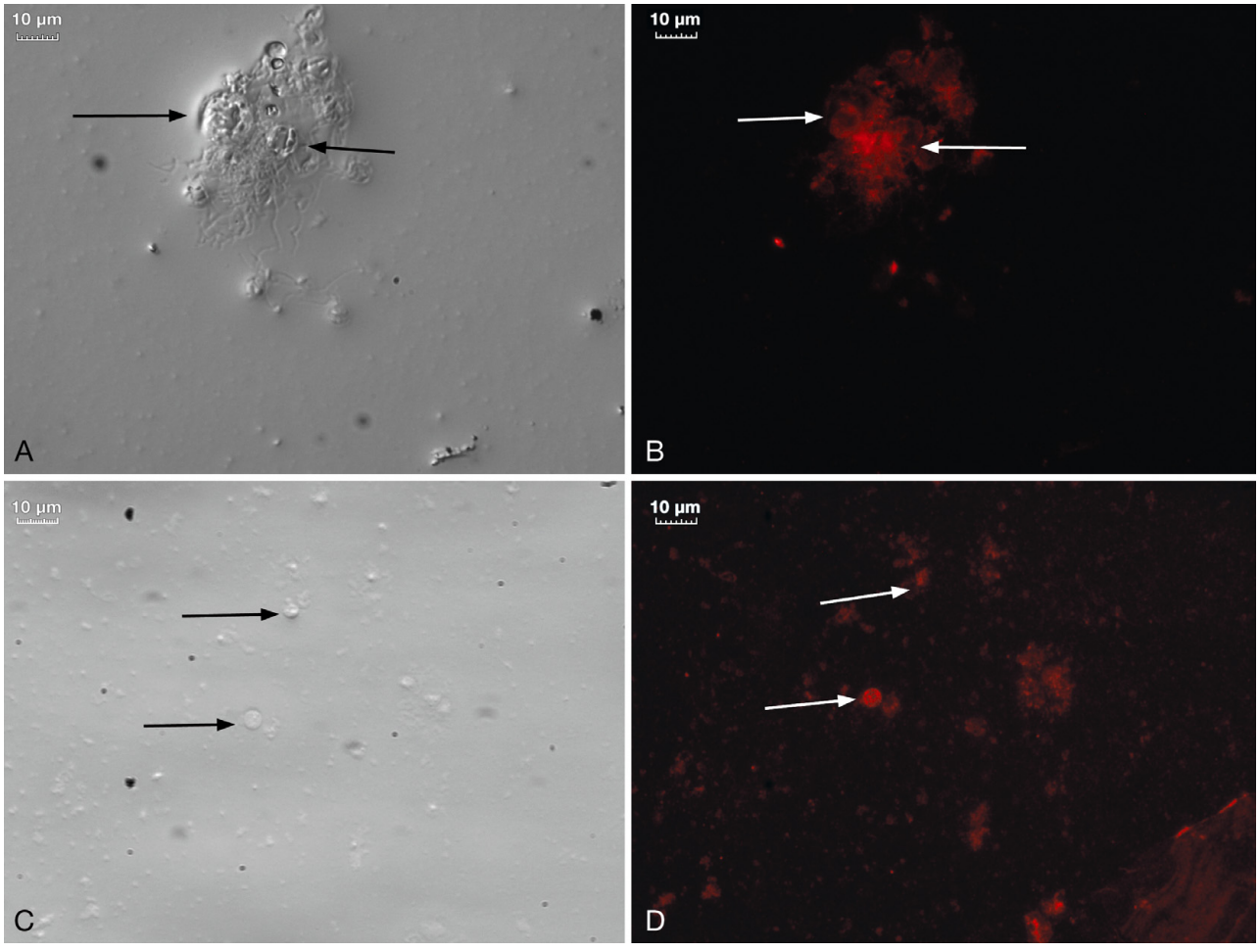

Fig. 5. *Batrachochytrium dendrobatidis* (arrows) after 5 d exposure to toad skin on TGhL agar (A and B) and TGhL agar alone (C and D). Fungal zoospores and sporangia were incubated with the *Aspergillus fumigatus* serine protease antiserum followed by a rhodamine-conjugated secondary antibody preparation. (A) and (C) indicate the presence of *Bd* sporangia (A only) and zoospores (A and C) using differential interference contrast microscopy. (B) and (D) show the same specimens using filters for red fluorescence detection

of enzymes into their environment, which resulted in tissue destruction. Elastin is a component of the extracellular matrix of vertebrates (Gosline & Rosenbloom 1984), and loss of cellular integrity may partially account for the symptoms of hyperkeratosis observed during chytridiomycosis. Gelatin is the hydrolyzed form of the extracellular matrix component collagen (Eastoe 1967) and can be used as a substrate when studying the presence of matrix metalloproteases. Casein is a model substrate that is often used in the study of proteases (North 1982). Our observation that casein serves as an attractant to *Bd* (Moss et al. 2008), as well as the work of Piotrowski et al. (2004) that showed that *Bd* can degrade casein, served as the motivation for using NFDM in this study. Our attempts to study protease activity in *Bd* using collagen or keratin as substrates did not yield enough material for broad-spectrum analysis of proteases. While we did observe gelatin hydrolysis on agar media, growth in liquid media was better supported with 1% NFDM.

Secretory inhibitors were added to *Batrachochytrium dendrobatidis* culture media before being assayed for elastolytic activity to investigate whether protease secretion could be blocked from an intracellular compartment. Brefeldin A, an antibiotic that interferes with anterograde transport from the endoplasmic reticulum to the Golgi apparatus (Nebenfuhr et al. 2002), affected elastolytic activity when added to *Bd* cultures 24 h prior to the assay. At a concentration of 10 µM, this antibiotic decreased secreted elastolytic activity by 26%. Monensin, an ionophore that interferes with Golgi-mediated vesicular traffic (Tartakoff 1983), decreased secreted elastolytic activity by 15% when added to *Bd* cultures in the same manner. The inhibition caused by Brefeldin A and monensin suggests that the secretion of proteases responsible for

elastolytic activity is a process mediated by the endoplasmic reticulum and Golgi apparatus in *Bd*.

The optimum pH for *Batrachochytrium dendrobatidis* cultures to produce extracellular protease activity was found to be approximately 7.5. Growth of *Bd* in culture is optimal between 6 and 7 (Piotrowski et al. 2004). Intracellular pH in frog skin is 7.2 (Duffey et al. 1986). Thus our observation that a pH of 7.5 is optimum for elastolytic protease production is relevant. Protein profiles of the culture supernatant revealed a complex polypeptide mixture. A combination of size-exclusion filtration and ion exchange chromatography was used to further purify the elastolytic activity observed in crude *Bd* supernatants. This partially purified (150 mM NaCl eluted fraction) material showed an 11-fold increase in elastolytic activity. This fraction was further characterized by assessing the effects of inhibitors and metal ions on enzyme activity. Elastolytic activity was greatly inhibited in the presence of EDTA, resulting in a 76% decrease in activity. This suggests a dependence of the activity on divalent cations. This observation is further supported by the enhanced activity observed with the addition of Ca^{2+} and Mg^{2+} ions. Inhibition of the activity by PMSF by 68% suggests that the enzyme is a serine protease. Partial inhibition by the elastase-specific inhibitor suggests that the elastolytic activity may be the result of more than one enzyme or of a multi-domain enzyme with characteristics of subtilases and serine proteases (Rao et al. 1998, Monod et al. 2002). Stimulation of elastolytic activity by Ca^{2+} is usually required by some proteases, such as subtilisin, for stability of the enzyme (Strausberg et al. 1995, Rao et al. 1998) and could possibly be used by *Bd* proteases for the same reason. Inhibition of elastase activity by Zn^{2+} ions was unexpected. Inhibition of activity by Zn^{2+} has been observed with rennin and HIV-associated protease, as well as coagulation factor VIIa proteolytic activity (Pedersen et al. 1991, Zhang et al. 1991).

Separation on SDS-PAGE showed the presence of 6 polypeptides in the purified material. Of these 6 polypeptides, 3 showed proteolytic activity when separated on casein and gelatin zymograms. These 3 proteins had estimated molecular weights of 110, 45, and 20 kDa. The 110 kDa band also showed immunoreactivity with the antiserum directed against *Aspergillus fumigatus* serine protease. Zoospores and sporangia grown on toad skin also displayed cross-reactivity when incubated with this antiserum. In an attempt to determine whether this activity was induced in the presence of toad skin, we analyzed zoospores and sporangia that were grown in the absence of toad skin, and they too showed cross-reactivity. These results suggest that *Batrachochytrium dendrobatidis* produces this protease at a basal level, although the activity can be enhanced *in vitro* with the presence of NFDM (presumably casein).

While the elastase activity described in this work does not fit perfectly into a single class or family of proteolytic enzymes (i.e. serine proteases, metalloproteases, aspartic proteases, etc.), it is likely that the activity was due to a protease with multiple substrates or more than one enzyme, for example the 46 kDa band seen in Fig. 4. The possibility of 1 or 2 gene expansions such as those seen in the dermatophytic fungi, *Microsporum* and *Trichophyton* (Brouta et al. 2002, Jousson et al. 2004), may also provide an explanation for this observation. The most prevalent proteolytic characteristics of the purified fraction deal with those associated with metalloproteases and serine proteases. The elastolytic activity from this material was markedly inhibited by a metal ion chelator. The expansion of the M36 family of metallopeptidases in *Batrachochytrium dendrobatidis* (Rosenblum et al. 2008) indicates the likelihood that some of these enzymes are responsible for the activity described here. The inhibitory effect of PMSF suggests that a serine protease is also responsible for some of this activity.

Possessing many different types of proteases or multiple forms of the same type of protease would make for a successful pathogen. Examples of pathogens already described as having this trait include the aforementioned dermatophytes, *Microsporum and Trichophyton* (Brouta et al. 2002, Descamps et al. 2002, Jousson et al. 2004), as well as *Candida albicans* (Hube et al. 1994). These pathogens can likely evade host defenses and establish infection more efficiently than those with a limited repertoire. Additionally, they would also be more resistant to mutations or attack by enzyme-specific inhibitors under this scenario. There exist many similarities between *Batrachochytrium dendrobatidis* and other pathogenic fungi, particularly dermatophytes and fungi that can degrade proteins such as those found in the extracellular matrix. Evaluating the mechanisms behind the pathogenesis of these organisms and using them as models to study *Bd* will provide a better understanding of how this organism is able to invade and destroy the epidermis of its amphibian host. Future studies on the activation of proteases and their contribution to the disease process as well as the characterization of other proteases produced by *Bd* are currently being evaluated in our laboratory.

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