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Production of extracellular proteolytic activity by *Histoplasma capsulatum* grown in *Histoplasma*-macrophage medium is limited to RFLP class 1 isolates

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Abstract

Extracellular proteolytic activity was studied for twenty-eight strains of *Histoplasma capsulatum* var. *capsulatum* and two strains of *Histoplasma capsulatum* var. *duboisii*. Secreted protease activity assessed by skim milk agarose clearance was limited solely to *H. capsulatum* var. *capsulatum* RFLP class 1 strains. There was a difference in proteolytic activity levels among class 1 strains. Extracellular proteolytic activity was further determined during growth of those strains in liquid medium using azodye-impregnated protein substrates. In general, the highest activities were measured when azocollagen was used, whereas azocasein and azoalbumin were cleaved less efficiently. The activity was inhibited by phenylmethylsulfonyl fluoride, 4-(2-aminoethyl) benzenesulfonyl fluoride, antipain and chymostatin, indicating, thereby, the presence of chymotrypsin-like serine proteases. Chromatographic analyses as well as variable substrate utilization at different culture times revealed production of at least two different enzyme pools of the same serine-like protease family. Our results demonstrate a distinctive ability of RFLP class 1 isolates to produce and secrete serine proteinase-type activity. This peculiarity may be relevant to the biology and pathogenesis of this particular clade of *H. capsulatum* isolates. Overall, the feature of extracellular proteolytic activity production enables a convenient and unequivocal identification of RFLP class 1 isolates and, thereby, can be used in *H. capsulatum* strain differentiation and typing.

Keywords

Histoplasma capsulatum; proteolytic activity; serine proteases

1. Introduction

Histoplasma capsulatum is an opportunistic fungal pathogen, which is the etiological agent of histoplasmosis. This common systemic human mycosis is endemic in certain areas of North and Latin America, but cases have been diagnosed worldwide. In the United States, most cases have been reported within the Ohio and Mississippi River valleys (Wheat, 2003). Progressive disseminated histoplasmosis is a severe respiratory and systemic disease, mostly of the reticuloendothelial system, manifesting itself in the lungs, bone marrow, liver, and the spleen. Most individuals infected with *H. capsulatum* experience an inapparent or benign influenza-like disease, however in severely immunocompromised HIV-infected patients the disseminated

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form of the infection occurs in 95% of cases and is mostly fatal if left untreated (Assi et al., 2006). While the immune system undoubtedly fulfils an important role in controlling histoplasmosis, features of the fungus itself contribute to the type and severity of disease.

Taxonomic studies based on either restriction endonuclease digestion patterns of DNA (Vincent et al., 1986, Spitzer et al., 1989, Kersulyte et al., 1992, Kasuga et al., 1999, Ueda et al., 2003) or analysis of selected gene loci (Keath et al., 1992, Kasuga et al., 2003) demonstrated a certain geographical constraint on the type of *H. capsulatum* strains that can be isolated. Restriction fragment length polymorphism (RFLP) allowed *H. capsulatum* isolates to be divided into at least six classes (Keath et al., 1992). This strain diversity does not only reflect geographical distribution, but also is related to virulence levels. It has also been proved that organisms with different genotypes inhabit the same geographic area. Isolates obtained from Latin and South Americas are grouped into RFLP classes 3, 5 and 6, whereas only one strain of class 4 has been isolated from soil in Florida so far (Eissenberg and Goldman, 1991). Strains of RFLP class 1 and 2 are prevalent in North America; however the latter are believed to be in preponderance. Class 1 organisms form a distinctive clade of strains in that they have been recovered from individuals having some degree of immunosuppression. In immunocompetent persons, RFLP class 1 strains may possibly establish inapparent yet persistent infections, which would be manifesting themselves pathologically only if the host subsequently became immunosuppressed (Eissenberg and Goldman, 1991). Interestingly, unlike class 2 strains, no soil isolate of RFLP class 1 has been yet reported. The inability to obtain class 1 organisms from the soil indicates their physiological and nutritional distinctness from other *H. capsulatum* clades.

In this study, we demonstrate a unique and distinctive ability of RFLP class 1 isolates to produce extracellular proteolytic activity. This feature will not be only useful in reliable identification of RFLP class 1 strains, but also suggests new possibilities for studies of *H. capsulatum* polymorphism and epidemiology.

2. Materials and Methods

2.1. Fungal strains

Strains of *H. capsulatum* used in this study are listed in Table 1. All strains were cultured from stock and maintained as yeast in liquid *Histoplasma*-macrophage medium (HMM) (Worsham and Goldman, 1988). Cultures were aerated by rotary shaking (150 rpm) at 37°C in 5% CO₂/95% air atmosphere.

2.2. Survey of extracellular proteolytic activity in *H. capsulatum* strains

Agarose-solidified HMM amended with 1.5% skim milk was used to detect extracellular proteolytic activity. Skim milk agarose was made as follows: 15 g of instant nonfat dry milk (Roundy's Inc., Milwaukee, WI) was reconstituted with 500 ml of distilled water and 5 g of agarose (SeaKem® LE Agarose, FMC Co., Philadelphia, PA) were added. The mixture was stirred thoroughly and autoclaved at 121°C for 25 min. Double-strength HMM (500 ml) was filter-sterilized and held in a water bath at 42°C. For plating purposes, HMM was poured into the warm sterile skim milk agarose bottle, mixed thoroughly and about 20 ml of the medium were poured per Petri dish plate. Such prepared plates were stored overnight at room temperature and then inoculated with a 10-μl droplet of log-phase HMM broth cultures of different *H. capsulatum* strains. Secreted proteolytic activity was assessed after 10 days of growth at 37°C in 5% CO₂/95% air atmosphere. The presence of proteolytic activity was observed as transparent clearance halos around fungal spots.

2.3. Preparation of culture supernatants

All strains of RFLP class 1 (Downs, UCLA531S, UWclin01, W#1, WU24, and CDC5568) were grown in liquid HMM for 7, 10 and 14 consecutive days. Cultures were aerated by rotary shaking (150 rpm) at 37°C in 5% CO₂/95% air atmosphere. Afterwards, cells were removed by centrifugation (3000×g, 5 min, 25°C). Cell-free supernatants (50 ml each) were then sterilized through 0.2 µm filters and partly concentrated and diafiltered against phosphate saline buffer (pH 7.2) using Vivaspin 20 units equipped with polyethersulphone 5-kDa nominal molecular mass cut-off limit membranes (Vivascience AG, Hannover, Germany). Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL), with bovine serum albumin as a standard.

2.4. Determination of proteolytic activity in culture supernatants

Proteolytic activity in culture supernatants was assayed using three different azodye-impregnated protein substrates: azocollagen (Chavira et al., 1984), azoalbumin (Tomarelli et al., 1949) and azocasein (Charney and Tomarelli, 1947). Digestion was done in 20 mM Tris/HCl buffer (pH 8.0) containing 10 mM CaCl₂. The amount of digested substrate was determined by measuring absorption at 520 nm for azocollagen or at 440 nm for azoalbumin and azocasein. One unit of specific proteolytic activity [U] defines a ΔA_{520} of 1.000 per one microgram of supernatant proteins per hour at pH 8.0 at 37°C using azocoll as a substrate. For azoalbumin and azocasein, one unit of specific proteolytic activity [U] defines a ΔA_{440} of 1.000 per one microgram of supernatant proteins per hour at pH 8.0 at 37°C. All protease substrates used in this study were purchased from Sigma (St. Louis, MO).

2.5. Susceptibility to protease inhibitors

0.1-ml supernatant aliquots were preincubated at 37°C for 1 h in 20 mM Tris/HCl buffer (pH 8.0) supplemented with 10 mM CaCl₂ and with individual protease inhibitors. Then azocollagen was added and reaction mixtures were incubated on a gyratory platform shaker at 37°C for 24 h. Control samples remained untreated with the inhibitors. The following inhibitors used at concentrations recommended by the manufactures were examined in this study: 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) (10 mM; Acros Chemicals, Geel, Belgium), phenylmethylsulfonyl fluoride (PMSF) (1 mM; Sigma), chymostatin (100 µM; MP Biomedicals, Solon, OH), antipain (100 µM; MP Biomedicals), leupeptin (100 µM; MP Biomedicals), E-64 (10 µM; Sigma), pepstatin A (1 µM; RPI Research Products, Mt. Prospect, IL), 1,10-phenanthroline (10 mM; Sigma), phosphoramidon (10 µM; MP Biomedicals), and bestatin (10 µM; MP Biomedicals). The inhibitory effect was expressed as percentage of remaining proteolytic activity in relation to untreated controls.

2.6. Chromatographic analysis

1.5-l UCLA531S cultures were grown in liquid HMM in 2.8-l Erlenmeyer flasks at 37°C in 5% CO₂/95% air atmosphere. Supernatants collected either from 7- or 14-day-old UCLA531S cultures were sterilized through 0.2 µm filters and partly concentrated by ultrafiltration using the Vivaflow 200 cassette unit equipped with a polyethersulphone 10-kDa nominal molecular weight cut-off limit membrane (Vivascience). A final concentration of high molecular weight fractions was achieved using Vivaspin 20 centrifugal filter devices equipped with polyethersulphone 5-kDa nominal molecular mass cut-off limit membranes (Vivascience), and the supernatants were diafiltered against 20 mM Tris/HCl (pH 8.0). The ÄKTA10purifier system (Amersham Biosciences AB, Uppsala, Sweden) equipped with a HiPrep™ 16/10 DEAE FF column was used. The column was initially equilibrated with 20 mM Tris/HCl (pH 8.0). Maximum 10-ml concentrated supernatant samples were loaded and proteins were eluted in a 0-40% gradient of 1.0 M NaCl in 10 column volumes at a flow rate of 5.0 ml per min.

Then, 2-ml fractions were collected and tested for proteolytic activity with azocollagen, azoalbumin and azocasein as described above.

3. Results

3.1. *H. capsulatum* strain screening for extracellular proteolytic activity

In our study, we have examined a panel of twenty-eight isolates of *H. capsulatum* var. *capsulatum* and two isolates of *H. capsulatum* var. *duboisii*. The strains used have been identified by using restriction fragment length polymorphisms (RFLP) of genomic DNA digested with BglII (Keath et al., 1992). In this method, digested DNA transferred to a nylon membrane was hybridized with the 1.85-kb HindIII-fragment containing the *YPS3* specific sequence. Using this approach, *H. capsulatum* strains could be divided into six different RFLP classes (Keath et al., 1992). In addition, these strains have also been typed using two recently developed *H. capsulatum* classification techniques based either on *YPS3* gene polymorphism (Bohse and Woods, 2007) or on fatty acid profile analysis (Zarnowski et al., 2007). The majority, but not all of the tested *H. capsulatum* var. *capsulatum* strains represented five of six recognized RFLP classes (Keath et al., 1992). Three isolates possessed, however, RFLP band patterns distinct from those observed in any of known *H. capsulatum* clades. The W#9 strain isolated from an AIDS patient had a RFLP profile similar to those seen in class 2, but contained also an additional unique band not observed in this class. Based on the *YPS3*-typing method (Bohse and Woods, 2007), this strain was classified as of class 2. The W#10 isolate, also obtained from an AIDS patient, represented a RFLP pattern completely different from those observed in any of known *H. capsulatum* clades. The last strain, named 69, was found related to Latin American group A (Kasuga et al., 2003) and to RFLP class 6 (Bohse and Woods, 2007), and had a pattern distinct from those observed in North American's class 1 or 2.

HMM/skim milk agarose was an effective medium to detect the extracellular proteolytic activity in *H. capsulatum* strains tested. The activity of secreted proteases manifested itself as clear transparent halos surrounding individual fungal colonies that became visible as soon as after a few days of incubation at 37°C in 5% CO₂/95% air atmosphere. Interestingly, the presence of extracellular proteases was observed only in *H. capsulatum* RFLP class 1 strains, whereas no extracellular proteolytic activity was detected in isolates of other RFLP classes even after a prolonged time of incubation (Fig. 1). Due to variability in growth rates and extracellular protease production among the strains (as shown in Fig. 1), we set 10 days as the standard incubation time for this plate assay.

3.2. Secreted proteases in culture supernatants

The extracellular proteolytic activity in cell-free culture supernatants of the class 1 strains was determined after 7, 10 and 14 days of growth in liquid HMM. Initially, we used skim milk as a protease substrate; however, this approach was found very inefficient and required enormously long incubation. Levels of the activity were, therefore, measured in culture supernatants using three different azodye-impregnated substrates: azoalbumin, azocasein and azocollagen. All class 1 strains were able to degrade these protease substrates, but showed some variation in the level of activity, which also varied during growth of individual isolates (Fig. 2).

The highest protease activities were observed when azocollagen was used (Fig. 2A). The maximal activity was detected in UCLA531S strain and this activity tended to increase during growth up to 77.5 ± 7.4 mU after 14 days of incubation. The proteolytic activity in supernatants from the remaining class 1 isolates was lower and no accumulation of this activity was observed. In UWclin01, CDC5568 and WU24 strains, levels of extracellular proteolytic enzymes were highest after 7 days of growth and then decreased. The activities in W#1 and

Downs culture supernatants reached the maximum 10 days after culture inoculation and then declined. Among all the strains, these two strains, W#1 and Downs, produced the smallest amounts of extracellular proteases.

Considerably lower extracellular protease levels were observed when measured with azocasein (Fig. 2B). Three different patterns of activity levels were observed. In UWclin01, CDC5568 and WU24 strains, the activity was the highest after 7 days of growth and then reduced. Similarly, 7-day-old UCLA531S and W#1 cultures also secreted amounts of proteases into the medium, but then prolonged growth led first to decrease and then to increase in the activity. In the case of Downs isolate, the general pattern of protease production was similar to this measured with azocollagen, but the overall activity was considerably smaller.

Relatively low activity levels of proteolysis were determined in the presence of azoalbumin; however, in this case only one pattern of the activity during culture growth was noticed (Fig. 2C). All the strains tended to increase the production of proteolytic enzymes reaching the highest level of 11.3 ± 3.4 mU and 12.1 ± 2.2 mU in 14-day-old Downs and UCLA531S culture supernatants, respectively.

3.3. Action of protease inhibition on extracellular proteolytic activity

In order to classify the secreted *H. capsulatum* proteases according to their catalytic cleavage mechanism, their sensitivity to classic protease inhibitors was evaluated. In this case, HMM broth without skim milk was used. In general, the same pattern of inhibition was observed in all the strains examined. No alterations in protease inhibition patterns in supernatants collected at different time points were observed. The results presented in Table 2 summarize the effect of protease inhibitors upon extracellular proteolytic activity in 7-day-old *H. capsulatum* class 1 cultures. No changes in extracellular protease activities were noted in the presence of the following inhibitors: bestatin, E-64, leupeptin, pepstatin A, 1,10-phenanthroline, or phosphoramidon. The cleavage of azocoll was completely blocked when AEBSF or chymostatin were added, whereas PMSF and antipain inhibited this activity partly.

3.4. Heterogeneity of extracellular proteases in UCLA531S strain

The accumulation of extracellular proteolytic activity in UCLA531S cultures prompted us to compare proteases secreted during growth of this strain. Supernatant samples collected after 7 and 14 days of growth were separated in ion exchange chromatography. Eluted fractions were examined for the presence of proteolytic activity using azocollagen, azoalbumin and azocasein.

Only one peak of activity was detected in fractions from the 7-day-old culture (Fig. 3A). The pattern of proteolytic activity in this fraction measured with azocollagen, azocasein and azoalbumin was coherent with the data shown in Figure 2. The major specific activity was determined in the presence of azocollagen, followed by azocasein and azoalbumin. Interestingly, the active pool did not bind to the column matrix and was immediately washed out from the column. As the supernatant samples were dissolved in 20 mM Tris/HCl (pH 8.0) buffer, we concluded the inability of eluted proteases to bind to the column bed was due to relatively high isoelectric point values of these proteins.

The analysis of 14-day-old supernatant also confirmed the presence of extracellular proteases in flow-through fractions; however an additional active protease pool was eluted later in a salt gradient (Fig. 3B). In this case, the binding of those eluted proteases to the column matrix indicated their isoelectric points to be lower in relation to the flow-through enzymes. Proteolytic activity was measured in both the flow-through and the salt-eluted fractions using the same set of three azodye-impregnated protease substrates. Overall, proteolytic activity measured in 14-day-old cultures was considerably higher than this determined after 7 days of

growth. There were some variations observed in proteolytic activity distribution between those two fractions. Comparing to younger cultures, the flow-through pool contained less proteolytic activity. In general, this fraction had the same substrate specificity and consisted mainly of azocollagen- (21.6 ± 3.2 mU), azocasein- (7.6 ± 1.0 mU) and azoalbumin-lytic (0.8 ± 0.3 mU) activities. The fraction eluted later in the salt gradient was considerably more proteolytically active and had a different substrate specificity profile. Indeed, this pool has been found responsible for accumulation of azocollagen- and azoalbumin-specific proteolytic activities in older UCLA531S cultures. The specific proteolytic activities in this pool were as follows: 55.6 ± 5.4 mU for azocollagen, 2.4 ± 1.6 mU for azocasein, and 11.2 ± 1.2 mU for azoalbumin, respectively.

Further experiments with protease specific blockers showed the same pattern of activity inhibition in both 7- and 14-day-old UCLA531S cultures (data not shown), indicating, thereby, the capability of *H. capsulatum* class 1 strains to produce and secrete different proteolytic enzymes of the same serine protease family during culture growth.

4. Discussion

The expanding population of immunocompromised individuals has focused attention on the importance of fungal pathogens and mycoses caused in these people. Many secreted enzymes produced by pathogenic fungi are considered important for infection in the host. Proteolytic enzymes are ubiquitous in occurrence in many mycopathogens and their crucial role in virulence of many fungal species has been described. Most of these studies concerned *Candida* spp. (Borg and Ruchel, 1988, Yamamoto et al., 1992, Vidotto et al., 2004), *Aspergillus fumigatus* (Jaton-Ogay et al., 1994, Iadarola et al., 1998) and *Cryptococcus neoformans* (Aoki et al., 1994, Chen et al., 1996, Ruma-Haynes et al., 2000, Vidotto et al., 2005); however there are many other species, like dimorphic fungi, which also cause serious infections in humans. Dimorphic fungi consist of six phylogenetically related ascomycetes: *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, and *Sporothrix schenckii*, but only few studies have shown proteolytic activity *in vitro* (Tsuboi et al., 1986, Sanada, 1987, Tsuboi et al., 1987, Cole et al., 1992, Carmona et al., 1995, Puccia et al., 1998, de Assis et al., 1999, Moon et al., 2006). There are also relatively few reports describing this type of activity in *H. capsulatum* (Okeke and Muller, 1991a, Okeke and Muller, 1991b, Muotoe-Okafor et al., 1996).

In this study, we surveyed a large number of *H. capsulatum* isolates for extracellular proteinase activity using a simple plate method that utilizes agarose-solidified skim milk/HMM. The plates inoculated with individual *H. capsulatum* strains were incubated for 10 days, but clear halos around fungal colonies resulting from proteolytic activity became visible as soon as after a few days of growth. Interestingly, we detected production of extracellular proteolytic enzymes solely in *H. capsulatum* var. *capsulatum* RFLP class 1 strains, whereas no such activity was observed in other RFLP classes of variety *capsulatum* (classes 3, 5 and 6 examined in this study) as well as in variety *duboisii*.

H. capsulatum class 1 strains have been speculated to establish inapparent, but persistent infection in immunocompetent persons, which may manifest itself only if the host subsequently became immunosuppressed (Eissenberg and Goldman, 1991). This class is prevalent along with class 2 in North America; however strains of this class are physiologically and metabolically distinctive isolates recovered from immunosuppressed individuals (Gass and Kobayashi, 1969, Medoff et al., 1986, Stone et al., 1990, Eissenberg et al., 1991), but - unlike class 2 strains - never from the soil. Concerning cell wall composition, class 1 represents chemotype II, which is observed also in Latin and South America isolates as well as in other varieties within this species. Chemotype II cell walls contain α -(1,3)-glucan and less chitin,

whereas chemotype I observed in class 2 strains has β -glucan and more chitin in cell walls (Eissenberg and Goldman, 1991). These explicit biochemical and physiological features along with distinct geographical distribution clearly emphasize a divergent character of the RLFP class 1.

Our finding is contradictory to earlier published studies demonstrating extracellular proteases in cultures of several strains of *H. capsulatum* var. *duboisii* and *H. capsulatum* var. *capsulatum* (Okeke and Muller, 1991a, Okeke and Muller, 1991b, Muotoe-Okafor et al., 1996). The discrepancies observed between these studies and our work are probably due to differences in methodology, especially in the media tested. Previous studies utilized various protein-supplemented media including modified 3M, glucose peptone salts or brain heart infusion agar (Okeke and Muller, 1991a, Okeke and Muller, 1991b, Muotoe-Okafor et al., 1996), whereas we grew *H. capsulatum* isolates on HMM/skim milk agarose or in HMM broth. Production of proteolytic enzymes is closely related to conditions influencing culture growth and development, which in turn are determined by the composition of nutritive media. Interestingly, HMM and other previously reported nutrient-rich media did not suppress the production of proteolytic enzymes, indicating, thereby, their constitutive character. On the other hand, the medium we used in our study enabled proteolytic activity production, but only in class 1 strains. None of the media used in other studies possessed this selective feature. A possible reason explaining those differences in protease secretion on various media might be that the other media may contain insufficient supply of certain nutrients, which obliges protease production. In a case of HMM, the medium provides nutritious constituents in amounts satisfactory to most of *H. capsulatum* classes, and in turn switches the proteases off. However, HMM/skim milk agarose does not fulfill the nutritional requirements of RFLP class 1 isolates and could suggest a reason for the production of extracellular proteases. Such observations have been made in relation to proteases secreted into different media by *C. albicans* (White and Agabian N, 1995) or *S. schenckii* (Tsuboi et al., 1988). Additionally, the initial pH of the media used in our and other studies could affect the production of proteolytic enzymes. For instance, White and co-workers (White et al., 1993) demonstrated that the levels of aspartyl proteases in *C. albicans* were controlled by medium pH. Unfortunately, the mechanism(s) triggering *H. capsulatum* to produce extracellular proteases remain(s) unidentified and for that reason are purely speculative at present.

The effects of inhibitors provided reliable information concerning the possible catalytic type of proteases secreted by *H. capsulatum* class 1 strains. The lack of inhibition in the presence of bestatin and pepstatin A ruled out the occurrence of aminopeptidases and aspartic proteases. The absence of any effects of phosphoramidon or 1,10-phenanthroline excluded the participation of metalloproteases. No inhibitory action of either E-64 or leupeptin ruled out the occurrence of cysteine proteases or trypsin-like serine proteases in *H. capsulatum* cultures. However, extracellular proteolytic enzymes were completely blocked in the presence of AEBSF or chymostatin, whereas PMSF and antipain only partly inhibited the activity. These inhibitors are specific to serine proteases; however the strong effect caused by chymostatin and the weak action of antipain precisely indicated the proteolytic enzymes in culture supernatants of *H. capsulatum* class 1 strains to be chymotrypsin-like serine proteases. Interestingly, protease inhibition patterns were similar in 7-, 10- and 14-day-old cultures, indicating the presence of serine protease over a range of *H. capsulatum* growth. This observation is in partial agreement with the study by Okeke and Müller (1991b), who postulated the presence of extracellular serine and metalloproteases in cultures of this dimorphic fungus. Similarly, our results concerning the nature of the investigated proteases might be considered consistent with the data presented by Muotoe-Okafor and co-workers (Muotoe-Okafor et al., 1996), who wrongly attributed the inhibitory action of chymostatin to aspartyl proteases. Interestingly, the elastolytic activity described by Okeke and Müller (1991a) was not inhibited by any of tested inhibitors, which might point out the presence of other elastin-specific

extracellular proteolytic enzymes. In fact, the use in our study of an elastase-specific inhibitor, elastinal, had no effect on the measured proteolytic activity (data not shown).

Subsequent chromatographic analyses of 7- and 14-day-old UCLA531S supernatants on a weak anion exchanger DEAE demonstrated the presence of serine proteases with alkaline pI in young and older cultures. The latter also contained an additional pool of serine proteases with neutral and/or acidic pIs, which were not observed in 7-day-old cultures. Such extracellular serine proteases have been previously characterized in other fungal species (Yuan and Cole, 1987). The identification of at least two different proteolytic activities of the same class in UCLA531S (Fig. 3) could account for the varying levels of proteolysis of different substrates at different culture times (Fig. 2). Indeed, patterns of substrate specificity in the flow-through remained fairly constant throughout the culture growth, but its overall proteolytic activity level has been reduced in older cultures. Unlike in this pool, the salt gradient-eluted protease fraction present only in those older *H. capsulatum* cultures had a considerably different substrate specificity profile. Similar variability was observed in all class 1 strains examined (Fig. 2), but we have not further delineated the proteases in other strains.

H. capsulatum lives and multiplies as a facultative intracellular pathogen in the host, which is different from most other pathogens. The secretion of proteases from *H. capsulatum* when it is either outside host cells or within host macrophages might be an important mechanism in the development of histoplasmosis. Our results showed that proteases of *H. capsulatum* could efficiently degrade collagen, which is one of the host structural barrier components and, thereby, might help the mycopathogen to infect and disseminate within the host. Extracellular proteases of *H. capsulatum* were also able to digest albumin and casein, and this fact may suggest their role in nutrient acquisition. The protease-assisted degradation of large proteins and concurrent release of small peptides and single amino acids would help to acquire nutrients essential for both fungal growth and metabolism; however, further studies will be necessary to verify these hypotheses as well as to determine expression status and investigate other possible functions of extracellular proteases in the pathogenesis of *H. capsulatum*.

Overall, the results presented in our study confirm the distinctiveness of class 1 isolates amongst other *H. capsulatum* groups. All of the tested *H. capsulatum* class 1 strains secreted proteolytic enzymes when grown on HMM/skim milk agarose or in HMM broth. All these strains were able to secrete different patterns of proteolytic enzymes of the same serine protease family with a broad substrate specificity. This peculiarity may be relevant to the biology and pathogenesis of this particular type of *H. capsulatum* isolates. The feature of extracellular activity production offers a convenient and unequivocal identification of RFLP class 1 isolates and, thereby, can be used in *H. capsulatum* strain differentiation and typing.

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Fig. 1.

Demonstration of extracellular proteolytic activity in *H. capsulatum* isolates. Fungal strains were cultured on agarose-solidified HMM amended with 1.5% skim milk. Secreted proteolytic activity visible as transparent clearance halos around fungal colonies was assessed after 10 days of growth at 37°C in 5% CO₂/95% air atmosphere. The presence of extracellular proteases was observed only in RFLP class 1 strains (here shown UCLA531S and Downs strains), whereas no activity was detected in other *H. capsulatum* classes (here represented by G217B of class 2 and G186B of class 3).

Fig. 2.

Determination of proteolytic activity during *H. capsulatum* class 1 culture growth in HMM broth. Proteolytic activity was assayed in W#1, Downs, UWclin01, CDC5568, WU24, and UCLA531S supernatants after 7 (open bars), 10 (single hatched) and 14 (double hatched) days of growth. The activity was determined with azocollagen (Panel A), azocasein (B), and azoalbumin (C).

Fig. 3.

Chromatographic assessment of extracellular protease heterogeneity during growth of UCLA531S strain. Supernatant proteins were separated in a 0-40% gradient of 1.0 M NaCl in 10 column volumes (long dashed line) on a HiPrep™ 16/10 DEAE FF column preequilibrated with 20 mM Tris/HCl (pH 8.0). Proteolytic activity was determined using azocollagen. Panel A. Chromatogram of extracellular proteins from a 7-day-old UCLA531S culture (solid line) with proteolytic activity found in flow-through fractions (solid black area). Panel B. Chromatogram of extracellular proteins from a 14-day-old UCLA531S culture (solid line) with proteolytic activities detected in flow-through fractions (solid black area) and also fractions eluted in the salt gradient (wide downward diagonally shaded peak).

Table 1
Fungal strains used in this study. *Histoplasma capsulatum* isolates used in this study

Strain	ATCC No. ^a	Source	Variant	RFLP class ^b	Comments
Downs	38904	clinical isolate, 1968, Missouri	<i>capsulatum</i>	1	
UCLA 531S		clinical isolate, California		1	
WU24		Broad Institute		1	
UWclin01		clinical isolate, Wisconsin		1	
W#1		AIDS patient, 1992		1	
CDC5568		clinical isolate, Virginia		1	
G217A	26031	clinical isolate, Louisiana		2	
G217B	26032	clinical isolate, Louisiana		2	
G222B	26034	clinical isolate, Louisiana		2	
W#2		clinical isolate, AIDS patient, 1992		2	
W#3		clinical isolate, AIDS patient, 1991		2	
W#6		clinical isolate, AIDS patient, 1992		2	
CDC5563		soil isolate, Virginia		2	
CDC5564		soil isolate, Virginia		2	
CDC5565		soil isolate, Virginia		2	
G184AR	26027	clinical isolate, Panama		3	
G186AR	26029	clinical isolate, Panama		3	
G186B	26030	clinical isolate, Panama		3	
UWclin02		clinical isolate, Wisconsin		3	
JHMA (H502)		clinical isolate, Sao Paulo, Brazil		5	
ERSC (H201)		clinical isolate, Sao Paulo, Brazil		5	
22-0453		clinical isolate, AIDS patient, 1992		6	
CFLA (H248)		clinical isolate, Sao Paulo, Brazil		6	
MSL (B1)		clinical isolate, Sao Paulo, Brazil		6	
MFG (H381)		clinical isolate, Sao Paulo, Brazil		6	
W#9		clinical isolate, AIDS patient, 1989		6	pattern like in class 2 with an additional distinct band not seen in this class
W#10		clinical isolate, AIDS patient, 1989		unassigned	a pattern never seen in other RFLP classes
69		infected bats, Houston Zoo		unassigned	related to Latin American group A (Kasuga et al., 2003) and is distinct from class 1 or 2
RV26821R	32281	clinical isolate, Belgium	<i>duboisii</i>		
UWclin03		clinical isolate, Wisconsin			

^a American Type Culture Collection.

^b *H. capsulatum* isolates were typed as described elsewhere (Keath et al., 1992; Bohse and Woods, 2007; Zarnowski et al., 2007).

Table 2
Inhibition of extracellular protease activity by protease specific inhibitors in 7-day-old culture supernatants of *H. capsulatum* class 1 isolates

	Percentage of remaining proteolytic activity ^a					
	W#1	Downs	UWclin01	WU24	CDC5568	UCLA531S
AEBSF	0	0	0	0	0	0
PMSF	57	71	84	80	75	43
Chymostatin	6	0	2	3	4	5
Antipain	74	82	97	92	91	80

^a Proteolytic activity was determined using azocollagen as described in Materials and Methods. Values are means of three independent determinations from a representative experiment.