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Extracellular Proteinase Activity of *Cryptococcus neoformans*

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Extracellular proteinase activity was studied for eight strains of *Cryptococcus neoformans* var. *neoformans* and two strains of *Cryptococcus neoformans* var. *gattii*. Proteinase activity was measured by protein agar clearance, azoalbumin hydrolysis, gelatin liquefaction, and protein substrate polyacrylamide gel electrophoresis. All strains of *C. neoformans* produced extracellular proteolytic activity. Maximal extracellular proteinase activity in supernatants of *C. neoformans* cultures was associated with late logarithmic- and stationary-phase cultures. *C. neoformans* was able to utilize murine immunoglobulin G1, bovine immunoglobulin G, and human complement factor 5 for growth in media containing these proteins as the sole sources of carbon and nitrogen, suggesting a capacity to degrade immunologically important proteins. Protein substrate polyacrylamide gel electrophoresis revealed several bands with proteolytic activity at apparent molecular masses of 200, 100, and 50 kDa. The results confirm the existence of extracellular proteinase activity for *C. neoformans*.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes life-threatening meningoencephalitis in 6 to 8% of patients with AIDS (9). Infection is acquired from the environment, presumably through the respiratory route (23). Little is known about the mechanism by which *C. neoformans* invades tissue and is disseminated from the lung. Studies of experimental infection in rats have shown that *C. neoformans* can penetrate the lung parenchyma within hours after being deposited in the alveolar space (12), suggesting that the fungus produces tissue-disrupting substances, such as proteolytic enzymes. A suggestion of in vivo protease production was obtained from histopathological studies of *C. neoformans* infection in beige mice in which collagen fibrils were degraded in infected tissues (34). However, the literature contains conflicting reports on the ability of *C. neoformans* to produce extracellular proteolytic activity: Staib (35) studied 32 strains on human serum agar plates and found no proteolytic activity; Federici (11) studied 7 strains and found proteolytic activity in only 2; Ahearn et al. (1) studied 8 isolates and found proteolytic activity in only 3; and Brueske (5) studied 1 strain and described extracellular proteolytic activity. More recently, two articles have reported proteinase activity by *C. neoformans* strains (2, 13).

Proteinase production has been implicated in the pathogenesis of several fungal (7) and bacterial (14, 19, 26; for a review of anaerobes, see reference 16) pathogens. Among fungi, two notable examples are *Candida albicans* (4, 10, 21, 22, 29) and *Aspergillus fumigatus* (18, 27, 28, 36, 37). In bacteria, immunoglobulin A (IgA) proteases have been associated with the virulence of *Neisseria gonorrhoeae, Streptococcus* spp., and *Haemophilus influenzae* (8, 24, 25, 33). Proteinases are believed to contribute to microbial virulence by destroying host tissues and digesting immunologically important proteins, such as antibodies and complement (19, 21, 26). In this study, we have revisited the question of whether *C. neoformans* cultures are associated with extracellular proteolytic activity. By employing a variety of tests for proteolytic activity, we established that all *C. neoformans* strains studied had associated extracellular proteolytic activity.

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MATERIALS AND METHODS

Strains. *Cryptococcus neoformans* var. *neoformans* ATCC 24067 and ATCC 52817 and *Cryptococcus neoformans* var. *gattii* ATCC 24065 and ATCC 24066 were obtained from the American Type Culture Collection (Rockville, Md.). *C. neoformans* var. *neoformans* NIH 371 was obtained from J. E. Bennett (Bethesda, Md.), cap 67 was obtained from E. S. Jacobson (Richmond, Va.), and J9A, J22, SB4A, and SB6A are recent clinical isolates (9). Serotype A is represented by strains SB4A, SB6A, and NIH 371; serotypes B and C are represented by ATCC 24065 (B) and ATCC 24066 (B/C), respectively; and serotype D is represented by ATCC 24067, J9A, and J22 (3). Strains were maintained in Sabouraud's dextrose agar (Difco Laboratories, Detroit, Mich.) at 4°C.

Determination of growth conditions. Strains were grown in media with various sources of carbon and nitrogen to determine the conditions that resulted in increased extracellular proteolytic activity. Ten-milliliter aliquots of culture supernatants were stored at −80°C, and the remainder were filtered and concentrated in Centriplus-10 concentrators (Amicon Inc., Beverly, Mass.) as per the manufacturer's instructions. All samples were stored at −80°C.

Growth curves. Cultures were grown in defined minimal medium (13 mM glycine, 29.4 mM KH_2PO_4, 10 mM $MgSO_4$, 3 μM thiamine, 15 mM glucose) supplemented in some experiments with 30 μCi of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml. For growth curve studies, 100-ml cultures were inoculated with approximately 100,000 organisms from an overnight starter culture. At each time point, samples were removed, fungal cells were counted with a hemacytometer, and the number of CFU was determined by plating on Sabouraud's dextrose agar. The samples were then pelleted, and the supernatant was stored at −80°C for later studies of proteolytic activity and gel electrophoresis.

Protein agar clearance. An assay involving clearance of turbidity in protein plates was developed for the measurement of proteinase activity. In a modification of a plate assay used by Odds and Abbott (30, 31), the following mixture was formulated: 1.5% agar, 15 mM glucose, 13 mM glycine, 29.4 mM KH_2PO_4, 10 mM $MgSO_4$, 3 μM thiamine, 0.1% azoalbumin (pH 4.5). For protein agar clearance, *C. neoformans* strains were plated on this agar and incubated at 30°C until clearance halos appeared. Alternatively, some plates were stained with 0.1% Coomassie blue R-250 in 40% methanol–10% acetic acid (Fisher Scientific, Fair Lawn, N.J.) for 30 min and destained with a solution of 40% methanol and 10% acetic acid. Lighter-staining areas surrounding colonies were interpreted as indicative of azoalbumin degradation.

Azoalbumin hydrolysis protease assays. The method of Charney and Tomarelli (6, 38) was used to screen for proteolytic activity in culture supernatants. Briefly, 2.5 mg of azoalbumin (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 2 ml of culture medium and/or buffer. The solution was gently mixed and then incubated in a 37°C water bath for 90 or 180 min. Protein hydrolysis was stopped by the addition of 8 ml of 5% trichloroacetic acid to precipitate proteins, and the solution was then filtered through 0.8-μm-pore-size Nalgene syringe filters

(Nalge Co., Rochester, N.Y.). Five milliliters of the filtrate was then mixed with 5 ml of 0.5 N NaOH, the solution was vortexed, and the A_{440} was measured on a Lambda 1 UV/visible-light spectrophotometer (Perkin-Elmer, Foster City, Calif.) or an Ultrospec 2000 UV/visible-light spectrophotometer (Pharmacia Biotech, Cambridge, England). Cells washed with sterile phosphate-buffered saline (PBS) were incubated in medium with 0.3% sodium azide. Cultures and supernatants were treated like those without azide, and azoalbumin hydrolysis assays were performed as described above. Calculations of proteinase activity were based on proteinase K (Boehringer Mannheim Corp., Indianapolis, Ind.) standards.

Gelatin liquefaction. The gelatin liquefaction assay was done in nutrient gelatin tubes (BBL, Becton Dickinson, Cockeysville, Md.) as per the manufacturer's instructions. Briefly, loopfuls of *C. neoformans* were inoculated into the nutrient gelatin tubes and incubated at 30°C. Liquefaction was observed by removing the tubes from incubation at 30°C and placing them at 4°C until the control uninoculated tube and other negative controls solidified (10 to 15 min). Proteinase production results in the liquefaction of nutrient agar.

Substrate PAGE. Nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize protease activity in gels and to determine the apparent molecular weight of the protease. Briefly, concentrated culture supernatants (~10-fold concentrated) were mixed with nonreducing PAGE sample buffer and electrophoresed on a 7.5% acrylamide gel with 0.1% gelatin (Sigma Chemical Co.) incorporated into the matrix by using the Mini-Protean II system (Bio-Rad, Hercules, Calif.) (15). Following electrophoresis, SDS was removed by washing the gel in two changes of 2.5% Triton X-100 (Sigma Chemical Co.). The gels were incubated for 72 to 120 h in a buffer containing 100 mM glycine, 2 mM $CaCI_2$, and 10 mM cysteine (pH 7.0) to allow renaturation and proteolytic digestion. The gels were then stained with 0.1% Coomassie blue R-250 and subsequently destained in a solution of 40% methanol and 10% acetic acid. Destaining revealed bands devoid of stain in sample lanes, indicative of protein digestion within the polyacrylamide gel.

Utilization of protein for growth. Growth of *C. neoformans* on different protein substrates was carried out in 96-well culture plates (Falcon; Becton Dickinson, Lincoln Park, N.J.). Approximately 200 CFU of sterile-PBS-washed *C. neoformans* in 50 μl was placed in each well. Bovine IgG, complement factor 5, albumin, casein (all from Sigma Chemical Co.), or protein G-purified murine anticryptococcal monoclonal antibody 2H1 diluted in sterile distilled H_2O was added for a final concentration of 0.1 mg/ml in 100 μl. All protein samples were filter sterilized through 0.45-μm-pore-size syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, Mich.). Twelve wells were used for each protein to allow multiple CFU determinations for each time point. The plate was left covered and wrapped at room temperature (20 to 25°C) to minimize evaporation. At each time point, samples were mixed, diluted, and plated on Sabouraud's dextrose agar. Colonies were counted after incubation for 48 to 72 h at 30°C. Statistical analysis of the data was performed by using the Kruskal-Wallis statistic (11a).

RESULTS

Extracellular proteolytic activity was studied as a function of culture age, hemacytometer fungal cell counts, and CFU counts (Fig. 1). The curve exhibits the expected lag, logarithmic, and stationary growth phases for microorganisms grown in culture media. Differences between hemacytometer yeast counts and CFU counts during the first 4 h suggest that some cells do not recover from the pelleting and washing steps prior to inoculation. Over the course of 240 h (10 days), there was no decrease in the number of viable organisms. Hemacytometer cell counts and CFU counts fell within the standard deviations of each other, which is consistent with little or no cell lysis. Proteolytic activity at the individual time points was determined by the azoalbumin hydrolysis assay. Activity was expressed as the amount of proteinase K per milliliter of supernatant which would be required to obtain the absorbance measured. There was little or no initial proteinase activity. Activity increased during logarithmic phase, such that the total activity peaked during stationary phase. Proteolytic activity per CFU (or per cell) during the exponential growth phase was 20 to 100 times greater than during stationary phase. Treatment of *C. neoformans* with 0.3% sodium azide killed the cells without obvious cell lysis, as determined by light microscopy. Azoalbumin hydrolysis assays revealed no proteolytic activity in supernatants of azide-killed cells after 24 h of incubation.

C. neoformans proteolytic activity was also studied by plating *C. neoformans* on minimal medium agar plates containing 0.1% azoalbumin. The plates are turbid yellow to orange because of the presence of the chromophoric azoalbumin. Incubation of these plates at 30°C resulted in the appearance of clearance halos around *C. neoformans* colonies (Fig. 2a). Clearance was also seen after 6 days by Coomassie blue R-250 staining (Fig. 2b); without staining, clearance was apparent after approximately 12 days. Surprisingly, there was also a gradual disappearance of the yellow color surrounding the colonies, suggesting that the bonds forming the azo linkage and responsible for the color had been either destroyed or modified. With slight variations in the conditions, opacities developed around colonies. Specifically, in a mixture containing 1.5% agar, 0.1% bovine serum albumin, and 15 mM glucose, with or without 0.5% gelatin, opacities around colonies were noted after 3 to 4 weeks (Fig. 2c). All 10 *C. neoformans* strains exhibited proteolytic activity when assayed by clearance of turbidity with the azoalbumin agar plate screen.

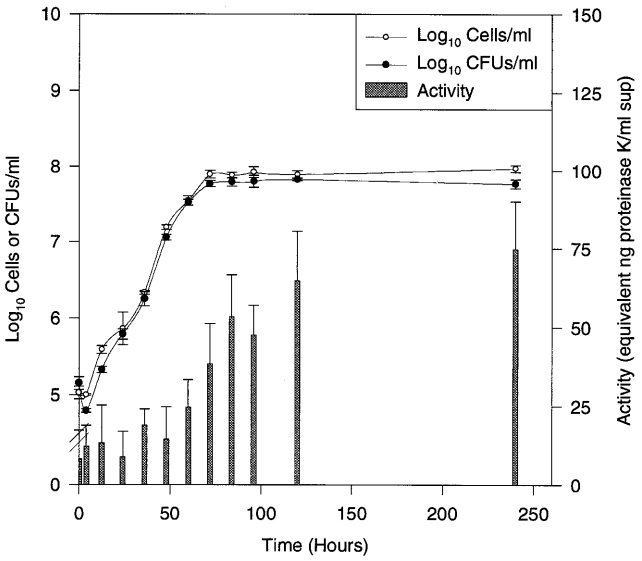

FIG. 1. Growth curve analysis of ATCC 24067 grown in defined minimal medium. Lines correspond to cell counts by hemacytometer and to CFU counts. Points and error bars show averages and standard deviations, respectively, of a minimum of four measurements. The bar graph depicts the amount of proteinase activity relative to a stock solution of proteinase K. Bars represent the averages and standard deviations of six measurements from three independent cultures. Bars for the 0- and 72-h time points show the averages and standard deviations of five measurements. (For time zero, one of six measurements yielded a value of 73 ng/ml. This value was not included in the average since it is an outlier which is 8 standard deviations from the mean of the other five measurements. For the 72-h time point, one measurement was lost.) This experiment was done three times, with similar results.

Gelatin liquefaction is a widely used method of testing proteinase activity in microorganisms (32). Proteases digest the gelatin and disrupt the gel matrix at room temperature, resulting in the liquefaction of the medium. Gelatin liquefaction assays were done three times for each strain, and all strains except NIH 371 liquefied gelatin consistently. Strain NIH 371 was tested four times but liquefied gelatin only twice.

Substrate gel analysis, or zymography, with gelatin as the substrate was also performed to detect proteinase activity. This assay is based on the fact that proteinases often retain activity after electrophoresis and are able to digest protein immobilized in the acrylamide gel. Following electrophoresis, the gels were incubated in a glycine buffer to allow renaturation and proteolytic activity to take place. Small areas devoid of Coomassie blue R-250 stain appeared in the gel matrix with molecular masses in the ranges of 200, 100, and 50 kDa (Fig. 3).

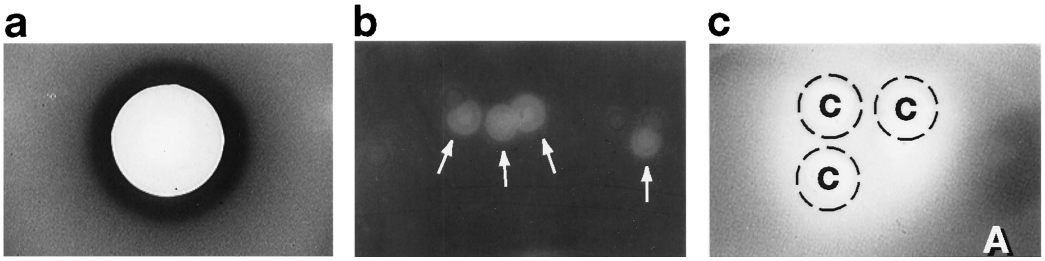

FIG. 2. Protein agar plates showing clearance halos or opacities indicative of protein degradation. (a) ATCC 24067 colony in agar with 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 15 mM glucose, 13 mM glycine, 3 μM thiamine, and 0.1% azoalbumin after 16 days of growth. (b) NIH 371 plated onto agar plates as described for panel a. Colonies were washed off the surface of the plate. The plate was then stained with 0.1% Coomassie blue R-250 for ~4 h and subsequently destained with 40% methanol–10% acetic acid. Lightly staining areas indicate the absence of the protein azoalbumin, suggesting that proteolytic digestion has taken place. Arrows point to areas lacking Coomassie blue staining. (c) ATCC 24067 grown on agar plates containing 1.5% agar, 0.1% albumin, 15 mM glucose, and 0.5% gelatin. "C" indicates a colony; "A" indicates normal agar.

The 200- and 100-kDa bands were observed in at least 10 separate substrate gel assays (data not shown). The 50-kDa band was weaker and was noted less frequently, but it was observed in at least three separate substrate gel assays (data not shown). Control experiments using chymotrypsin revealed clearance at the apparent molecular mass of 25.5 kDa, in good agreement with the molecular mass of chymotrypsin. Autoradiography of polyacrylamide gels, with and without copolymerized protein substrate, with [^{35}S]methionine-labeled culture supernatants revealed protein bands in regions corresponding to gelatin clearance (data not shown).

Figure 4 shows the growth of *C. neoformans* in solutions with protein as the sole source of nutrients. The proteins used were albumin, casein, the IgG1 monoclonal antibody 2H1 to capsular polysaccharide, complement factor 5, and bovine IgG. Of the conditions studied, *C. neoformans* grew best in minimal media but was also capable of growth in solutions of the purified murine monoclonal antibody 2H1, human complement factor 5, bovine IgG, albumin, and casein. While growth in these solutions was significantly lower than growth in minimal media, it was considerably higher than that observed in sterile distilled H_2O or PBS (data for the latter not shown).

Table 1 summarizes the level of proteinase production by *C. neoformans* under different growth conditions. Proteolytic activity was low in all conditions tested, and none of the conditions increased proteolytic activity above that found in minimal media.

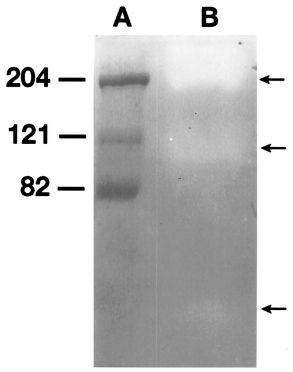

FIG. 3. Substrate gel electrophoresis in a 7.5% acrylamide–0.1% gelatin gel of concentrated culture supernatants. Lanes: A, molecular mass markers (Bio-Rad, Inc.); B, ATCC 52817. Arrows mark discrete zones of gelatin clearance. The lowest arrow indicates a weak zone of clearance with an apparent molecular mass of 50 kDa.

DISCUSSION

Many yeasts and fungi are known to secrete extracellular proteases (for a review, see reference 32). For *C. neoformans*, some investigators have reported extracellular proteolytic activity (2, 5), whereas others have detected no activity (35). This discrepancy may be attributable to the conditions used for eliciting and measuring proteinase activity. Brueske (5) and Aoki et al. (2) have described proteolytic activity in culture supernatants. Goodley and Hamilton (13) have recently reported the isolation of a 200-kDa protein with serine protease activity. In this study, we have used protein agar clearance, azoalbumin hydrolysis, gelatin liquefaction, and protein substrate electrophoresis to demonstrate proteinase activity associated with *C. neoformans*. Furthermore, we demonstrated that *C. neoformans* can utilize biologically important molecules as the sole sources of carbon and nitrogen for growth.

Proteolytic activity in cell supernatants can be the result of the secretion of extracellular proteinases and/or the release of intracellular proteinases as a result of normal cell growth and/or cell death. Distinguishing between these two sources of proteinase activity can be difficult and often requires isolation and molecular characterization of the enzyme. Our studies cannot distinguish between these two sources of proteolytic activity. Nevertheless, several observations suggest that the proteinase activity measured in each study was not a result of cell lysis. First, a prolonged study of the correlation of proteinase activity with hemacytometer cell counts and CFU counts revealed a relatively constant total proteolytic activity without major changes in numbers of cells or CFU. Second, proteolytic activity was present in the phases of culture growth corresponding with the period of logarithmic growth. Third, growth of *C. neoformans* in protein agar plates was accompanied by hydrolysis of protein in agar, which is consistent with the release of extracellular proteinases. Fourth, azoalbumin hydrolysis assays using azide-killed cultures incubated at 30°C for 24 h showed no proteolytic activity. Azide-treated cells were visible under light microscopy and detectable by hemacytometer counting. However, CFU plating on Sabouraud's dextrose agar did not result in any colonies, indicating that the cells were killed. Together, the data suggest that proteolytic activity is not likely to be due to leakage from damaged or dead cells (data not shown). Considering that many other fungi produce extracellular proteinases and that *C. neoformans* is a free-living organism which must obtain nutrients from environmental sources, the most straightforward interpretation of our results and those of others (2, 5, 13) is that *C. neoformans* produces extracellular proteinases.

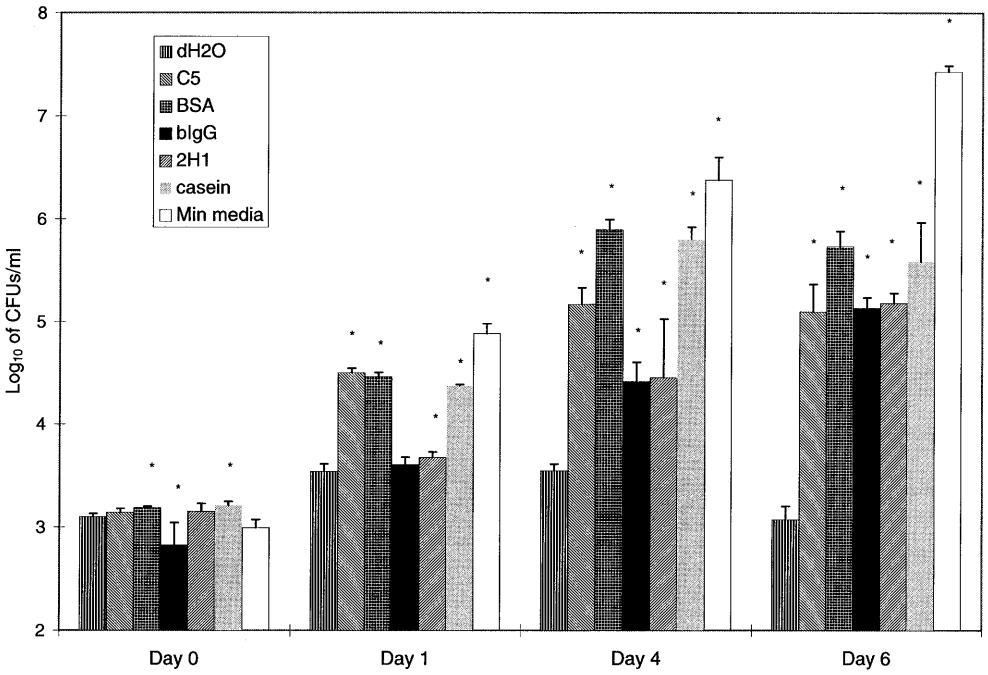

FIG. 4. Growth of ATCC 24067 in solutions containing 0.1 mg of protein per ml as a source of carbon and nitrogen. The negative control was sterile distilled water (dH_2O). The positive control was minimal (min) media. Protein sources were complement component C5 (C5), bovine serum albumin (BSA), bovine IgG (bIgG), monoclonal antibody 2H1, and casein. All protein samples were filter sterilized prior to addition. The experiment was done twice with all protein sources except complement factor 5, with similar results. Bars represent averages and standard deviations of a minimum of four measurements. *P* values were calculated by using the Kruskal-Wallis statistic relative to growth in dH_2O, and comparisons for which *P* is <0.05 are marked by asterisks.

Because of the conflicting data in the literature, we used four methods to test for proteinase activity. Azoalbumin hydrolysis is rapid and utilizes cell-free supernatant cultures but is relatively laborious. This assay is dependent upon the hydrolysis of azoalbumin into small peptides soluble in 5% trichloroacetic acid with a maximum at A_{440} (6, 38). If the proteinase cleaves only a few bonds within the protein sequence, leaving large trichloroacetic acid-precipitable peptides, a false-negative result may be obtained. The second method, gelatin liquefaction, relies on proteinases degrading collagen, the major component of gelatin. While simple, this assay has a major drawback in that for *C. neoformans*, it may take weeks before a result is definitive. Protein agar clearance is a classical method used to screen live colonies for proteolytic activity (30, 31). One interesting phenomenon that appears in some protein agar cultures is the development of opacities around colonies instead of clearance. This phenomenon has been described by others (20) and appears to be dependent on the type of protein, salt concentrations, and other growth conditions. The opacification phenomenon presumably reflects the precipitation of hydrolysis products in the agar. Protein gel opacification has been documented for yeasts in various reports (20). Substrate gel analysis is effective in visualizing proteolytic activity. Band clearance zones provide evidence for proteolytic activity in the gel and approximate molecular masses for proteinases.

TABLE 1. Summary of growth conditions and relative proteolytic activity of ATCC 24067

Carbon source[a]	Nitrogen source[b]	Activity[c]
G	Gly	++++
None	0.5% BSA	++
None	0.5% casein	+++
None	Gly	+++
R	Gly	++++
M	Gly	++++
Ac	Gly	++
Cit	Gly	+++
Ala	Gly	++
Val	Gly	+++
Leu	Gly	+++
Ile	Gly	++
None	Gly	++
Ac	1% peptone	+++
Ac	None	+
M	1% peptone	++++
M	BSA	+++

[a] G, 15 mM glucose; R, 15 mM raffinose; M, 15 mM mannitol; Ac, 15 mM acetate as sodium acetate; Cit, 15 mM citrate as sodium citrate; Ala, 13 mM alanine; Val, 13 mM valine; Leu, 13 mM leucine; Ile, 13 mM isoleucine.

[b] Gly, 13 mM glycine; BSA, bovine serum albumin.

[c] Proteolytic activity, as a percentage of activity with minimal medium, is summarized as follows: +, 1 to 30%; ++, 31 to 60%; +++, 61 to 90%; ++++, > 90%.

Substrate gel analysis of concentrated *C. neoformans* culture supernatants revealed discrete bands of proteinase activity. At least three bands with proteolytic activity, with apparent molecular masses of 50, 100, and 200 kDa, were observed in substrate gels. Since protein substrate gel analysis omits mercaptoethanol reduction and the boiling of protein samples in SDS to preserve proteolytic activity, the molecular masses calculated relative to standards are approximate. A recent report suggests that the molecular mass approximations made by this method could exceed the actual molecular masses by 15 to 20% (17). The three discrete bands in supernatants of *C. neoformans* cultures indicate either the presence of multiple proteolytic enzymes or the occurrence of degradation (both of which could result in fragments which retain proteolytic activ-

ity) or both. Proteolytic activity was very weak, and we attempted to produce solutions with high proteolytic activity by concentrating culture supernatants or by ammonium sulfate precipitation. These efforts were unsuccessful, which is consistent with the lability and/or self-digestion of the enzyme(s). The observation of a 200-kDa protein with proteolytic activity is consistent with the report of Goodley and Hamilton (13).

Rapid tissue invasion by *C. neoformans* has been observed in experimental rat pulmonary infections (12). Collagen fibril and extracellular matrix degradation has been observed in experimental murine cryptococcal infections (34). These in vivo observations are suggestive of and consistent with the elaboration of tissue-degrading enzymes, such as extracellular proteinases, by *C. neoformans* (12, 34). In this article, we report *C. neoformans* extracellular proteinase activity and demonstrate that this fungus can grow in the presence of immunoglobulin or complement proteins as the sole sources of carbon and nitrogen. These results suggest the need for further studies to determine the role, if any, of proteinases in virulence.

ACKNOWLEDGMENTS

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