Mycopathologia **128:** 143–150, 1994.

Extracellular proteolytic activity of *Cryptococcus neoformans*

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Received 17 May 1994; accepted in revised form 6 October 1994

Abstract

Eight strains of *Cryptococcus neoformans* var. *neoformans* isolated from AIDS patients in the Infectious Disease Institute, University of Turin, Italy, were examined for growth and extracellular proteolytic activity in culture with solid and liquid media. All of the strains grew well on Yeast Carbon Base (YCB) agar medium supplemented with both 0.1% (w/v) bovine serum albumin (BSA) and 0.01% (w/v) polypeptone (Pp), and produced a clear proteolytic zone around their colonies, whereas they exhibited less growth and proteolytic activity on YCB medium supplemented with BSA alone. Strain #8 with a strong proteolytic activity was cultured in three different liquid media. Its growth was limited in YCB medium supplemented with 0.1% BSA, but was moderate in that with 0.01% Pp. Enhanced growth was supported by the addition of both BSA and Pp to the YCB medium. The relative value of the final cellular yields obtained with the above YCB-0.1% BSA, YCB-0.01% Pp and YCB-0.1% BSA-0.01% Pp media was approximately 1:10:20. In the culture with YCB medium containing both BSA and Pp, a rapid decrease in the amount of BSA was demonstrated by a spectrophotometric assay and gel electrophoresis of the culture supernatant after the log-to-stationary phase. The proteolytic activity in the culture supernatant became detectable after the log phase when tested with skim milk agarose plates. These results allowed us to conclude that *Cr. neoformans* var. *neoformans* is able to secrete protease and to utilize protein as a source of nitrogen.

Key words: Bovine serum albumin, *Cryptococcus neoformans*, Growth, Nitrogen source, Protease secretion

Introduction

Cryptococcus neoformans is the etiological agent of human and animal cryptococcosis. Reflecting the increasing diffusion of AIDS, the risk of cryptococcal infection in immunocompromised hosts is arousing the attention not only for its virulence factors but also in respect to the biological and biochemical characteristics of the organism [1, 2]. Comprehensive studies by Kwon-Chung et al. [3–7] have demonstrated that at least the following three cellular characteristics are closely associated with the virulence and pathogenicity of this fungus: the ability to grow at 37 °C, to produce a polysaccharide capsule, and to express phenoloxidase activity responsible for melanin synthesis (see also a review article by Polak [8]).

In the other medically important fungal group, *Candida* species, Staib [9] first reported the extracellular proteolytic activity of *Candida albicans* in 1965. Ever since much experimental information, which indicates that extracellular acid proteinase is the most plausible virulence factor for this fungus, has been published [10, 11]. In contrast to *C. albicans*, *Cr. neoformans* is usually considered to be nonproteolytic [9, 12] and only a few studies have focused a great deal of interest on its proteolytic activity [13, 14].

In the present study, we examined the growth and extracellular proteolytic activity of clinical isolates of *Cr. neoformans* by using solid and liquid media containing bovine serum albumin (BSA). We showed that this fungus could utilize BSA as a source of nitrogen for its growth.

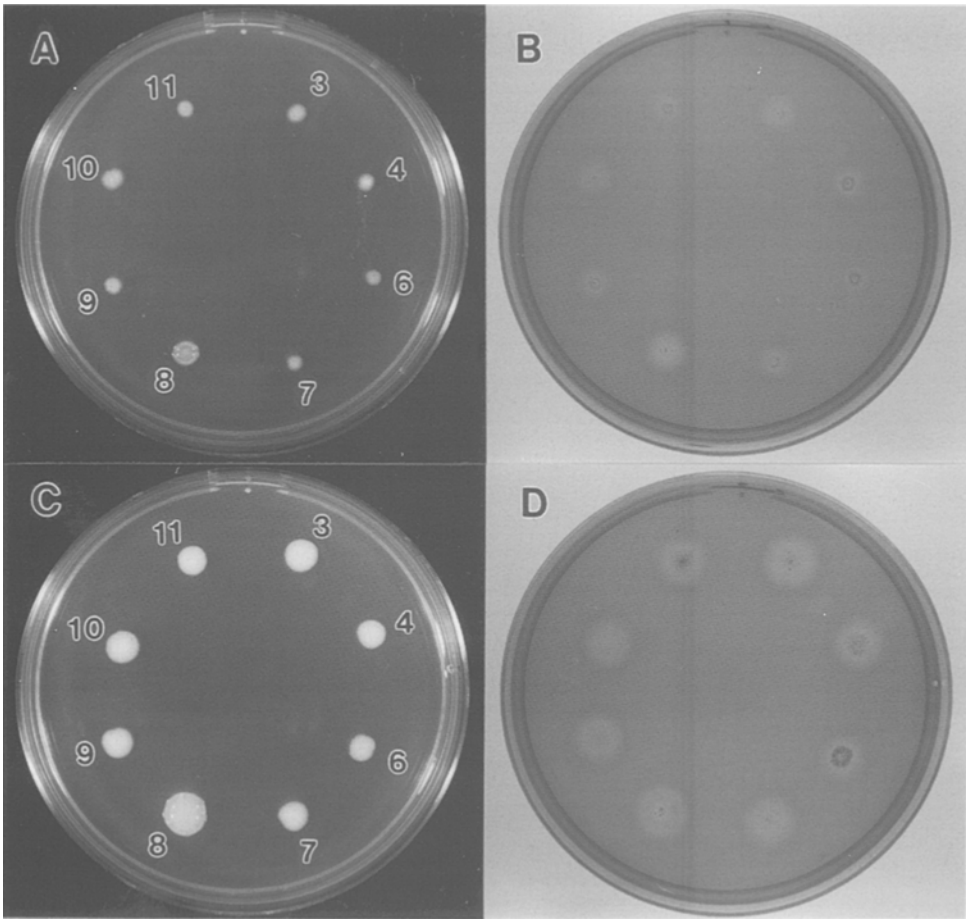

Fig. 1. Growth and proteolytic activity of *Cr. neoformans* isolates on YCB-BSA (A and B) and YCB-BSA-Pp (C and D) agar after 14 days of incubation at 30 °C. Photographs were taken before (A and C) and after (B and D) staining with CBB. Fungal cells were washed away during fixation of the agar plates with TCA. The strain numbers are shown on the plates A and C.

Materials and methods

Fungal strains

Cr. neoformans #3 (AS128DSL), #4 (AS152), #6 (AS125RC), #7 (AS134MD), #8 (AS127MH), #9 (AS122CR), #10 (AS121GL) and #11 (AS120PS) were used in the present study. These strains were isolated from AIDS patients hospitalized in the Infectious Disease Institute, University of Turin, Italy. All the strains were positive for urease, produced melanin on DOPA agar medium [15], but did not grow on canavanine-glycine-bromothymol blue (CGB) agar medium [16]. Serotyping of the isolates was performed by a slide aggulutination test with a commercially available kit, Crypto Check (Iatron Lab. Inc., Tokyo, Japan), according to the procedures recommended by the supplier. Only strain #6 belonged to serotype A and the others were shown to be serotype D [17]. Therefore, all the isolates were identified as *Cr. neoformans* var. *neoformans*. These strains were maintained on Sabouraud dextrose agar slants at 5 °C.

Culture on agar media

Yeast Carbon Base (YCB) (Difco Lab., Detroit, MI, USA) agar medium supplemented with 0.1% (w/v) BSA or 0.1% BSA plus 0.01% (w/v) polypeptone (Pp) were used to examine protease secretion by *Cr. neoformans*. Solutions of 11.7% (w/v) YCB, 1% (w/v) BSA (fraction V; Sigma Chem., St.Lous, MO, USA), and 1% BSA plus 0.1% (w/v) Pp (Wako Pure Chem., Osa-

ka, Japan) were sterilized by filtration through a membrane filter of 0.2 μm pore size. To prepare 200 ml of medium, 20 ml of the YCB solution were added to 160 ml of 1.88% (w/v) melted agar at 56 °C, and then 20 ml of the BSA or BSA plus Pp solution were added to the mixture. Twenty ml portions of the media thus prepared were poured into Petri dishes; these media were referred to as YCB-BSA and YCB-BSA-Pp, respectively. A very small amount of cells of each strain, grown on Sabouraud dextrose agar at 30 °C for 3 days, was inoculated on the YCB-BSA and YCB-BSA-Pp agar plates with needles. After incubation at 30 °C for 14 days, the plates were fixed with 10% (w/v) trichloracetic acid (TCA) for 2 hours and then stained with Coomassie brilliant blue G-250 (CBB). Protease production was detected by the appearance of proteolytic zones around the colonies.

Culture in liquid media

In addition to liquid YCB-BSA and YCB-BSA-Pp, YCB supplemented with 0.01% Pp (YCB-Pp) was also used in this experiment. Cells of strain #8, grown on Sabouraud dextrose agar at 30 °C for 3 days, were collected by centrifugation and washed three times in sterile distilled water. The washed cells were then transferred to 150 ml of each medium in flasks to give an initial optical density of 0.02 at 550 nm and cultured at 30 °C with shaking. After 8, 20, 32, 48, 72, 96 and 120 hours, the growth of the cells was monitored with a spectrophotometric method and reading the optical density at 550 nm. Culture supernatants, after centrifugation at 8000 rpm for 15 min, were used as samples for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), quantitative measurements of BSA and detection of proteolytic activities.

SDS-PAGE

Culture supernatants prepared as above were electrophoresed in 10% (w/v) polyacrylamide gel containing 0.01% (w/v) SDS at 20 mA for 3 hour according to the method of Laemmli [18]. Fifty μl portions of each culture supernatant were used per well. After running, the gel was fixed with 10% TCA, stained with CBB and destained in distilled water. A kit of marker proteins (BioRad Lab., Richmond, CA, USA) was used for molecular weight determinations in SDS-PAGE.

Measurement of the amount of BSA

The amount of BSA remaining in the culture supernatants was estimated by differences in the absorbance at 280 nm measured before and after precipitation of BSA with TCA. Two hundreds μl of 20% TCA were added to 400 μl of culture supernatants in Eppendorf tubes. The tubes were kept on ice for 30 min and then centrifuged at 10000 rpm for 15 min. The absorbance of the supernatant fractions following centrifugation was measured at 280 nm. The reduction in the absorbance by the precipitation with TCA was referred to the amount of BSA. The absorbance value 0.66 at 280 nm for 1% (w/v) solution of BSA was used to calculate the amount of BSA [19].

Proteolytic activitiy in culture supernatants

Skim milk agarose plates [20] were used to detect proteolytic activity in the culture supernatants. A 2% (w/v) solution of skim milk (Difco Lab.) in 0.1 M sodium acetate buffer (pH 5.3) was mixed with an equal volume of 2% (w/v) melted agarose (Standard Low Mr; BioRad Lab.) at 56 °C, and 20 ml of the mixture were poured into Petri dishes. Culture supernatants were lyophilized and 17-fold concentrated samples were prepared. Ten μl of the samples was spotted onto the skim milk agarose plates. After incubation at 37 °C for 20 hours, the plates were fixed with TCA and stained with CBB to examine the production of transparent spots.

Results

Growth and protease production on agar media

In this experiment, YCB was supplemented with 0.1% BSA, or 0.1% BSA plus 0.01% Pp. The basal YCB contained a total amount of amino acids (histidine, methionine and tryptophane) at an extremely low concentration, 5×10^{-4}% (w/v) [21]. Thus, the BSA and Pp used at the above concentrations could be regarded as the sole source of nitrogen.

Growth and protease production of the 8 clinical isolates on solid media are shown in Fig. 1. All of the 8 isolates grew slowly on YCB-BSA and produced a small proteolytic zone around their colonies (Fig. 1A and B). On YCB-BSA-Pp, good growth and the proteolytic activities of the strains were observed (Fig. 1C and D), showing digestion and utilization of BSA

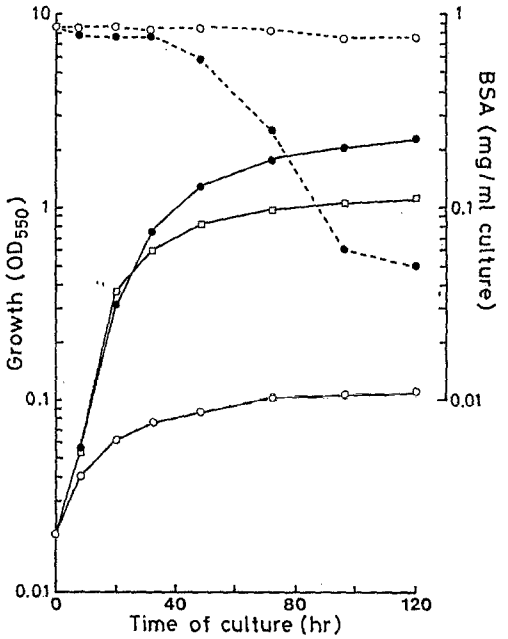

Fig. 2. Growth of *Cr. neoformans* strain #8 (solid lines) in liquid YCB-BSA (○), YCB-Pp (□) and YCB-BSA-Pp (•) media at 30 °C, and changes in the amount of BSA (dotted lines) in YCB-BSA (○) and YCB-BSA-Pp (•) media.

for growth. Standard strains of *Cr. neoformans* var. *neoformans* (serotypes A, D and AD) and var. *gattii* (serotypes B and C), which were provided by the Research Center for Medical Mycology, University of Teikyo School of Medicine, were also examined as controls. YCB-BSA-Pp, as compared with YCB-BSA, well supported the growth of the 5 serotypes. Among the 5 serotypes, serotypes B and C formed large colonies. Protease production by serotypes B and C was more extensive than that of serotypes A, D and AD on both agar media (data not shown).

Similar results in respect to growth and protease production were obtained when BSA in the above two media was replaced with soluble casein.

Growth and protease production in liquid media

As can be seen in Fig. 1, strain #8 showed the strongest proteolytic activity among the 8 strains tested. Thus, this strain was used for further examination. The growth curves obtained with this strain in YCB-BSA, YCB-Pp and YCB-BSA-Pp are shown in Fig. 2. As can be seen in this figure, the growth of strain #8 was very low in YCB-BSA while moderate growth was observed in YCB-Pp. The growth of this strain in YCB-BSA-Pp did not differ from that in YCB-Pp in its early stages but exceeded the growth in YCB-BSA in its later stages. The relative values of final cellular yields obtained with the above three media were estimated approximately as 1:10:20 by absorbance at 550 nm (Fig. 2). The difference in the final cellular yield of strain #8 between YCB-Pp and YCB-BSA-Pp was considered to be due to the difference in the utilization of BSA by the strain as a source of nitrogen for its growth.

Changes in the amount of BSA in YCB-BSA and YCB-BSA-Pp cultures are also shown in Fig. 2. A very slight decrease in the amount of BSA was found in the former culture. In contrast, a rapid decrease in the amount of BSA was induced after 48 hours in the latter. These results are in agreement with the growth curves in YCB-BSA and YCB-BSA-Pp. They also indicate degradation and utilization of BSA in YCB-BSA-Pp.

SDS-PAGE of culture supernatants

The supernatants prepared from YCB-BSA and YCB-BSA-Pp cultures of strain #8, as shown in Fig. 2, were subjected to SDS-PAGE. Supernatants from the YCB-Pp culture were not electrophoresed because no protein bands were observed in SDS-PAGE in preliminary trials. Fig. 3 shows the SDS-PAGE profiles of protein in the culture supernatants. In the culture with YCB-BSA, no clear changes in the patterns of protein bands were observed between the supernatants sampled at 0 and 120 hours, thus showing no or little utilization of BSA in the medium. In contrast, a decrease in the staining intensity of the BSA band was evident in the YCB-BSA-Pp culture as the incubation time increased. These results are well consistent with the changes in the amount of BSA measured by the spectrophotometric method shown in Fig. 1.

In a separate culture with YCB-BSA-Pp of strain #8, the culture supernatant was sampled after 11 days of growth and electrophoresed (Fig. 4). The BSA band disappeared almost completely (lane b). To examine the appearance of minor bands, the supernatant was concentrated 10-fold by lyophilization and electrophoresed. The staining intensity of the main BSA band of the concentrated sample (lane c) was clearly lower than that of the supernatant sampled at 0 hour (lane a). Nevertheless, many minor bands, smaller in molecular size than the main BSA band, became detectable by concentration. This result suggested that

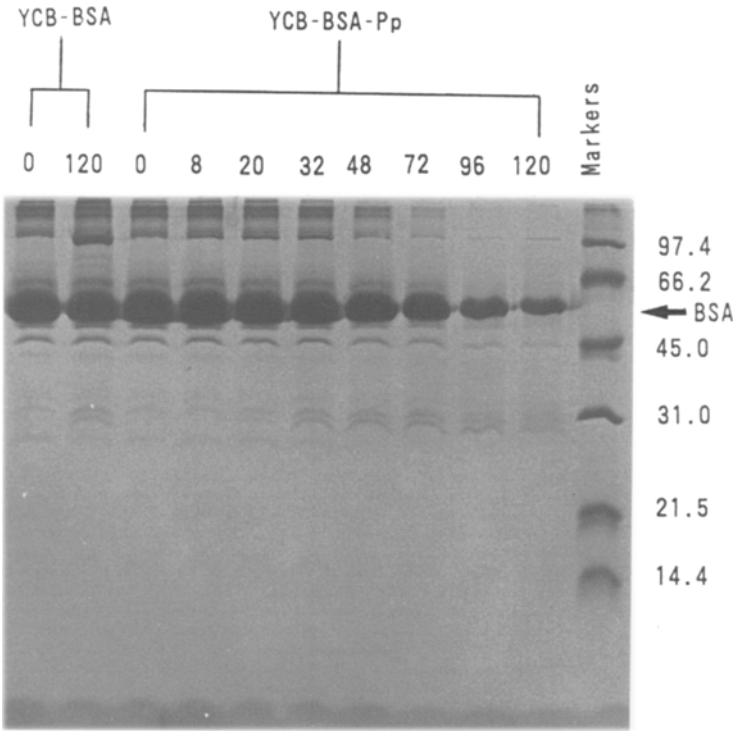

Fig. 3. SDS-PAGE profile of proteins in the supernatant of YCB-BSA and YCB-BSA-Pp cultures in the experiment shown in Fig. 2. Growth time (hour) is shown on each lane. The molecular weight (kD) of the marker proteins is shown on the right.

the degradation of BSA was clearly induced during the prolonged incubation period.

Proteolytic activity in culture supernatants

In preliminary trials, we attempted to follow the changes in the proteolytic activity secreted into supernatant during cultivation in YCB-BSA-Pp by a spectrophotometric method. This activity, however, was too weak to assay spectrophotometrically even though the samples had been concentrated by lyophilization. Thus, a casein clotting method with skim milk agarose plates [20, 22] was adopted for our purpose. Aproximately 17-fold concentrated supernatants from the culture shown in Fig 2 were spotted onto the plate and incubated at 37 °C for 20 hours. Although clotting of casein unfortunately was not observed with all the samples, transparent spots due to casein digestion on the plate were observable after staining with CBB (Fig. 5). The transparent spots were produced with the concentrated culture supernatants sampled after 20 hours of the growth. In contrast, transparent spots were not detected on the skim milk agarose plates with supernatants from cultures grown in YCB-BSA and YCB-Pp (data not shown).

Discussion

Little attention has been paid to the proteolytic activity of *Cr. neoformans* because this fungus is usually regarded as being nonproteolytic [9, 12]. Müller and Sathi [13] first demonstrated the ability of *Cr. neoformans* to degrade or split up 2 out of a total of 13 human

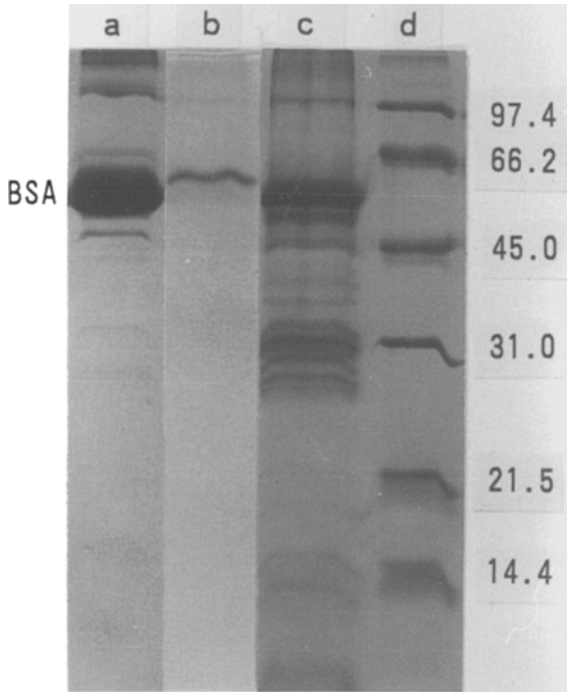

Fig. 4. SDS-PAGE profile of proteins in supernatant from prolonged culture in YCB-BSA-Pp. *Cr. neoformans* strain #8 was grown at 30 °C for 11 days, and the supernatants sampled on days 0 and 11 were compared. Lane a, supernatant on day 0; lane b, supernatant sampled on day 11; lane c, 10-fold concentrated supernatant on day 11; lane d, marker proteins. The molecular weight (kD) of the marker proteins is shown on the right.

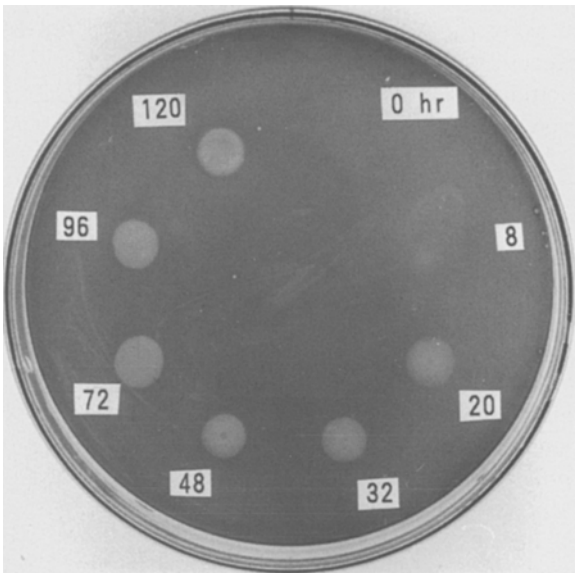

Fig. 5. Detection of proteolytic activity in culture supernatants on skim milk agarose. Supernatants were sampled from the YCB-BSA-Pp culture of *Cr. neoformans* strain #8 shown in Fig. 2. They were concentrated by lyophilization and spotted on the skim milk agarose plate. After incubation at 37 °C for 20 hours, the plate was stained with CBB to detect transparent spots due to digestion of casein. The numbers in the figure indicate growth time of the culture (hour) at which the supernatants were sampled.

plasma proteins tested in their immunoelectrophoretic study. Brueske [14] reported that a clinical isolate of *Cr. neoformans* var. *neoformans* secreted extracellular proteases into its culture supernatant. The protease production was maximal when the isolate was grown in an essential salts solution supplemented with BSA as the sole source of carbon and nitrogen. It was repressed, however, by supplementation with easily utilizable ammonium sulphate and glucose.

In the present study, we investigated the growth and induction of proteolytic activity in clinical isolates of *Cr. neoformans* var. *neoformans* using solid and liquid media supplemented with BSA as an inducer. All of the clinical isolates did not grow efficeintly and produce distinct proteolytic activity on solid and liquid YCB media supplemented with BSA alone. This result was contrary to that reported by Brueske [14] in which BSA was a potent inducer of extracellular proteases. In our study, efficient growth and proteolytic activity were observed when YCB was supplemented with both BSA and a very small amount of easily utilizable Pp. This result suggests that the rapid growth depended on Pp in the early stages of development is important for the later efficient production of proteolytic activity.

In the case of proteinase-positive *Candida* species, the accumulation of BSA degradation products in culture supernatant has been demonstrated by gel electrophoresis [23-25]. In the present study, although SDS-PAGE profiles showed a clear decrease in the amount of BSA in YCB-BSA-Pp cultures in accordance with the results obtained with a spectrophotometric assay, degradation products of BSA were not detected without concentration of the culture supernatants. Furthermore, any protein band, which was expected to be protease secreted by the fungus, also was not found in the gel. The most plausible explanation for our results may well be that protease production under our conditions was too small to be detected in SDS-PAGE, and that consequently the degradation products of BSA in a limited amount are immediately utilized for fungal growth before they can accumulate in the culture supernatant.

We could not utilize a spectrophotometric method to assay proteolytic activity in culture supernatants because of its low activity. Thus, we used a casein clotting method that has been reported to be 100-fold more sensitive than spectrophotometric methods and to be capable of detecting less than 1 ng of proteinase [20, 22]. Casein clotting on the skim milk agarose plate unfortunately was not observed either in the unconcentrated or 17-fold concentrated supernatants from cultures on YCB-BSA-Pp. Proteolytic activity, however, in the concentrated culture supernatants was detected as transparent spots on the plate by staining the casein with CBB. According to Foltmann et al. [20], clotting depends on cleavage of a phenylalanine-methionine bond in casein. Thus, protease produced by *Cr. neoformans* may have low activity against that bond, thus failing to produce clotting of casein.

In conclusion, our results demonstrated that *Cr. neoformans* can secrete protease under certain conditions. Purification and characterization of the extracellular protease of *Cr. neoformans* are the subjects of our continuing investigations. Recently, Goodley and Hamilton succeeded in isolating an extracellular high molecular weight (200 kD) proteinase from culture filtrate of *Cr. neoformans* (Abstracts of 2nd International Conference on *Cryptococcus* and Cryptococcosis, p. 108, Sept. 1993, Milan). The enzyme was shown to be a Ca^{++} Jand Mg^{++} dependent serine protease with an optimal pH at 7.5–8.5. Therefore, it is of great interest whether the extracellular protease of *Cr. neoformans* is associated with its virulence and pathogenesis in cryptococcal infections.

Acknowledgements

We wish to express our thanks to Dr L. Ajello, Department of Ophthalmology, Emory University School of Medicine, Atlanta, GA, USA, for critically reading the manuscript. We are also grateful to Dr H. Yamaguchi, Research Center for Medical Mycology, Teikyo University School of Medicine, Tokyo, for supplying the serotype strains and to Mr N. Wada, Iatron Laboratories Inc., Tokyo, for providing the Crypto Check kit. This study was supported in part by a Grant-in-Aid (no. 06671839 to S.A.) from the Ministry of Education, Science and Culture of Japan. A part of this work was presented at 2nd International Conference on *Cryptococcus* and Cryptococcosis held in September 1993 in Milan.

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