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Induction of secretory acid proteinase in *Candida albicans*

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Candida albicans **and some other pathogenic** ***Candida*** **species, when grown in a medium containing a protein as a sole source of nitrogen, secrete an acid proteinase. Culture supernatants were assayed for proteinase activity, and were also analysed by Western blotting with antibodies raised and affinity-purified against proteinase of** ***C. albicans*****. Proteinases secreted by** ***C. tropicalis*** **and** ***C. parapsilosis*** **were antigenically related to that of** ***C. albicans*****, but had different molecular masses. The proteinases secreted by** ***C. lipolytica*****,** ***C. rugosa*** **and** ***C. lusitaniae*** **were not antigenically related. The kinetics of proteinase secretion by** ***C. albicans*** **were monitored by activity and by Western blotting. With BSA as the nitrogen source, proteinase secretion increased exponentially until about 16 h. Culture supernatants of BSA-grown cultures accumulated proteinase to about a 1000-fold higher level than those of ammonium-sulphate-grown cultures.** ***In vivo*** **labelling experiments showed that proteinase was not detectably accumulated in the cells, but was secreted immediately after synthesis. Immunoprecipitation of** ***in vitro*** **translated poly(A)-containing RNA identified a putative pre-protein of about 54 kDa. As well as BSA, other proteins (haemoglobin, ovalbumin, histone), peptone and tryptone, when used as nitrogen sources, could induce proteinase, but to different levels. When Casamino acids or an amino acid mixture (equivalent to the composition of BSA) was used as nitrogen source, no induction was observed. Ammonium sulphate, or any other ammonium salt, repressed secretion of proteinase.**

Introduction

Candida albicans is recognized as the most important pathogenic yeast, causing candidosis in immunocompromised humans and other warm-blooded animals. There has been an increasing incidence of candidosis in the last two decades, attributed to the widespread use of antibiotics and immunosuppressive agents (Odds, 1988). Among various hydrolytic enzymes reported as possible virulence factors in the pathogenicity of *C. albicans*, secretory acid proteinase has gained substantial importance, and its significance in candidosis has been studied by several groups (Staib, 1969; Remold *et al.*, 1968; Ruchel, 1981; MacDonald & Odds, 1980; Ray and Payne, 1990). The physiology of induction of secretory acid proteinase in *C. albicans*, under one set of conditions, has been studied by Ross *et al.* (1990). The ability of other species of *Candida* to secrete proteinases, and the immunological relatedness of these proteinases has also been reported (Ruchel *et al.*, 1983, 1986).

Using a very sensitive assay for proteinase, and Western blot analysis employing affinity-purified anti-proteinase antibodies, we studied proteinase secretion by different *Candida* species, and the immunological relationships of these proteinases to that of *C. albicans*. We also studied the kinetics of proteinase induction in *C. albicans*, and induction by different proteins, peptone and tryptone. A putative pre-protein of proteinase was identified by *in vitro* translation of RNA isolated from induced cells and immunoprecipitation.

Abbreviations: CS, culture supernatant; AS, ammonium sulphate.

Methods

Organisms and growth conditions. C. albicans SC5314 was obtained from Dr D. R. Kirsch (Squibb Institute of Medical Research, Princeton, New Jersey, USA). *C. albicans* ATCC 10261 was obtained from Dr M. G. Shepherd (University of Otago, Dunedin, New Zealand), *C. tropicalis* 3113 and 3118, *C. parapsilosis* 3323, *C. rugosa* 3467, *C. lusitaniae* 3484, *C. lipolytica* 3229, *C. utilis* 3469, *C. krusei* 3518, *C. shehetae* 2500 and *C. guilliermondii* 3126 were obtained from the National Collection of Industrial Microorganisms (NCL, Pune, India). These were maintained on YPD-agar [1% (w/v) yeast extract (Difco), 2% (w/v) peptone, 2% (w/v) glucose and 2% (w/v) agar]. For all experiments, peptone from Glaxo (India) was used, unless otherwise mentioned. All minimal media contained 0·17% yeast nitogen base without amino acids and without ammonium sulphate (Himedia, Bombay, India) and 2% (w/v) glucose (autoclaved); to this, either

ammonium sulphate (AS) or BSA was added to a final concentration of 0·2% from 4% (w/v) filter-sterilized (BSA) or autoclaved (AS) stock solution. When a mixture of three amino acids (1 mg histidine-HCl, 2 mg DL-methionine and 2 mg DL-tryptophan per litre) was added to the above media, they are referred to as M1-AS or M1-BSA; otherwise they are referred to as M2-AS or M2-BSA. A loopful of yeast cells from freshly prepared YPD-agar slants was grown in liquid YPD medium at 30 °C with shaking for 14 h (OD_{595} reached approximately 9·0). This preculture was diluted to 1% (v/v) in experimental media, and incubated at 30 °C on a gyratory shaker at about 200 r.p.m. Cell number was calculated from OD_{595} calibrated using a haemocytometer. Buds were counted as separate cells.

Proteinase assays. Proteinase activity was checked either by milk agarose spot assay or spectrophotometrically. The composition of milk agarose was 1% (w/v) agarose, 1% (w/v) skim milk powder, and 0·114 M-sodium acetate, pH 5·3 (Foltmann *et al.*, 1985). Culture supernatants (CS) or their dilutions, (5 µl each) were spotted on the milk agarose plate. Doubling dilutions of purified proteinase or CS of fully induced BSA broth were spotted to serve as standards. The plate was covered with Saran wrap and incubated at room temperature. Proteinase activity was seen as a white spot due to clotting of casein. The diameter of spots of standards and samples were measured after 6 to 24 h incubation at room temperature. A linear relationship was obtained when the diameter (linear scale) was plotted against the amount of enzyme (logarithmic scale), from which the amount of enzyme in unknown samples was calculated (Schumacher & Schill, 1972). In the spectrophotometric assay, proteinase activity was determined by the extent of digestion of BSA at pH 3·6, which was derived from the amount of TCA-soluble material found after incubation of BSA with proteinase (Shimizu *et al.*, 1987). A change of one A_{280} unit in 30 min at 37 °C was taken as one unit of enzyme activity.

Purification of proteinase. Secretion of proteinase was induced by growing *C. albicans* SC5314 in M1-BSA. The proteinase was purified by a modified method of Shimizu *et al.* (1987). About 850 ml CS, after its pH was raised from 4·6 to 5·0 with 100 mM-trisodium citrate, pH 8·3, was applied to a DE52 column (1·5 × 29 cm) which had been equilibrated with 10 mM-sodium citrate, pH 5·0 (Buffer A). After the column was washed with one bed volume of buffer A, it was eluted with a linear gradient of 0 to 1 M-KCl in buffer A. Fractions displaying activity were pooled, dialysed against buffer A, and bound to a DEAE-Sepharose CL-6B column (1 × 15 cm) equilibrated in the same buffer. Proteinase was eluted by a decreasing pH gradient generated by passing an acidic buffer (4 mM each of DL-aspartic acid, L-glutamic acid and glycine, pH 2·6) through the column. The proteinase was purified to apparent homogeneity as assessed by SDS-PAGE using the discontinuous buffer system of Laemmli (1970). It had a specific activity of over 100 A_{280} units (mg protein)$^{-1}$.

Affinity purification of anti-proteinase antibodies. Antibodies were raised against purified proteinase in a rabbit. Affinity purification was done essentially as described by Iwaki *et al.* (1989). The purified antibodies were stored in TBS (20 mM-Tris, pH 7·6, 150 mM-sodium chloride) containing 0·1% NaN_3, at 4 °C. For Western blot analysis (Towbin *et al.*, 1979) these antibodies were used at 1:900 dilution in TBST (TBS with 0·05% Tween 20). Immunodetection was done using the protoblot alkaline phosphatase system (Promega).

RNA Isolation. RNA was isolated by a modified method of Chomczynski & Sacchi (1987). The modifications were: (1) a higher concentration (1·25×) of solution D was used to process more cells in a smaller volume; (2) glass beads were used to break the cells; and (3) the final RNA pellet was washed in 3 M-sodium acetate, pH 5·5, to remove any DNA contaminants (Hereford & Rosbash, 1977). Poly $(A)^+$ RNA was isolated by one cycle of purification on oligo(dT)-cellulose column as described by Maniatis *et al.* (1982), except that SDS was omitted from all the buffers, and before eluting the bound RNA, the column was washed only with column loading buffer (20 mM Tris/HCl, pH 7·6, 0·5 M-NaCl and 1 mM-EDTA).

In vivo labelling and immunoprecipitation. To check the specificity of immunoprecipitation, an M2-BSA grown culture (10 h) of *C. albicans* strain SC5314 was labelled *in vivo* with 50 µCi (1·85 MBq) ml^{-1} of [^{35}S]-methionine (>800 Ci (2·96 × 10^{13} Bq mmol^{-1}, Amersham) for 20 min. The labelling reaction was stopped by adding TCA to 10% (w/v) final concentration. For pulse-chase, 100 µCi [^{35}S]methionine was added to 1 ml M2-BSA culture and incubated for 4 min. Then unlabelled methionine was added to a final concentration of 200 µg ml^{-1} to initiate the chase. Samples were taken out at different time intervals, added to prechilled Eppendorf tubes and centrifuged immediately. For preparation of crude extracts, pellets were ground with glass beads (0·3 g glass beads (0·45 mm) to 0·1 g pellet) in 0·3 ml grinding buffer (50 mM-Tris/HCl, pH 8·0, 1 mM-EDTA, 1 mM-PMSF and 1 mM-β-mercaptoethanol), by vortexing for 6 min, in 1 min bursts, with cooling on ice in between. Crude extracts prepared in this way and supernatants were processed separately for immunoprecipitation.

For immunoprecipitation, the method of Anderson & Blobel (1983) was essentially followed. Preadsorption of antibodies to protein A-Sepharose beads was done as described by Kessler (1981), and preclearing was done as described by Ausubel *et al.* (1987). The immunoprecipitates were resolved by SDS-PAGE, and radiolabelled bands were detected by fluorography (Chamberlain, 1979).

Inducers other than BSA. Haemoglobin, ovalbumin, histone, peptone, tryptone, Casamino acids [4% (w/v) stock in water], and amino acid mixture [2% (w/v) stock, equivalent to composition of BSA] were each added separately to M2 medium at 0·2% final concentration as a sole nitrogen source. At various times, CS was collected and analysed by milk agarose spot assay and by Western blot.

Results and Discussion

Proteinase secretion by Candida species

Proteinase secretion by various medically important *Candida* species and *S. cerevisiae* was determined by milk agarose spot assay (Fig. 1). This assay can detect less than 1 ng of proteinase, and it is about 100-fold more sensitive than the spectrophotometric assay (unpublished results). Most of the pathogenic *Candida* species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. rugosa*, *C. lusitaniae*, *C. lipolytica*) secreted proteinase when grown with BSA as a nitrogen source. *C. utilis*, *C. krusei*, *C. shehatae* and *S. cerevisiae* did not show any activity (Fig. 1). Culture supernatants of all these species were also analysed by immunoblotting with *C. albicans* anti-proteinase antibodies. Proteinase of *C. tropicalis* and *C. parapsilosis* were antigenically related to that of *C. albicans* (Fig. 2). However, the apparent molecular mass of proteinase secreted by *C. albicans* was 45 kDa, whereas that of *C. tropicalis* was 49 kDa and that of *C. parapsilosis* 36 kDa (Fig. 2). The secretory acid proteinases of these three species have been found to be related,

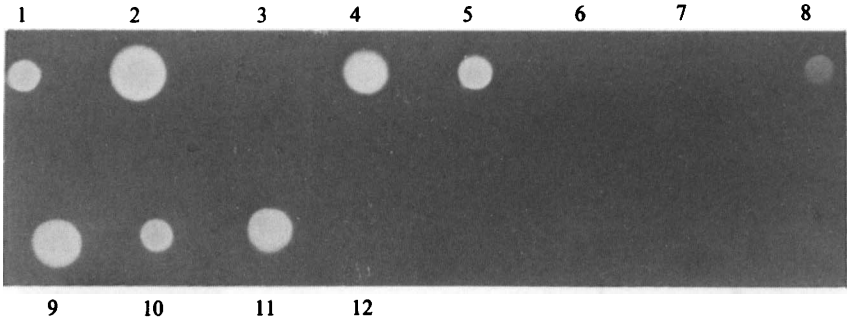

Fig. 1. Milk agarose spot assay for secretory acid proteinase. Different strains were grown in BSA broth for 48 h, and 5 μl of the culture supernatants were spotted on a milk-agarose plate and incubated at room temperature for 6 h. Diameter of the spots indicates proteinase level. The yeast strains used were 1, *C. albicans* 3466; 2, *C. albicans* 3471; 3, *C. krusei* 3518; 4, *C. lusitaniae* 3484; 5, *C. rugosa* 3467; 6, *C. utilis* 3469; 7, *C. shehatae* 3500; 8, *C. guilliermondii* 3126; 9, *C. lipolytica* 3229; 10, *C. parapsilosis* 3323; 11, *C. tropicalis* 3118; and 12, *Saccharomyces cerevisiae* 3511.

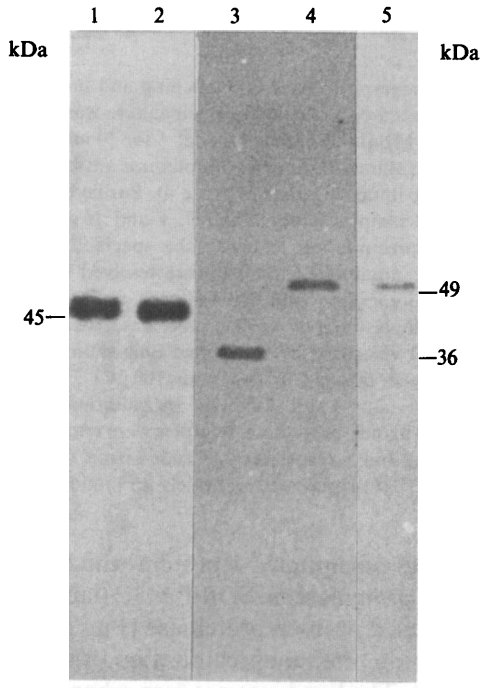

Fig. 2. Western blot analysis of culture supernatants of various yeast strains grown in BSA broth. Affinity-purified anti-proteinase antibodies raised against *C. albicans* proteinase were used. Lanes: 1, *C. albicans* SC5314; 2, *C. albicans* 10261; 3, *C. parapsilosis*, 3323; 4, *C. tropicalis* 3118; 5, *C. tropicalis* 3113.

partially related, or unrelated, depending on the antiserum employed (Ruchel *et al.*, 1983, 1986). Among pathogenic *Candida* species, *C. albicans* and *C. tropicalis* are considered to be very similar, and *C. parapsilosis* as the next most similar (Odds, 1988). This view is further strengthened by our Western blot data.

Kinetics of proteinase induction in C. albicans

A YPD preculture of *C. albicans* strain SC5314 was diluted into M1-AS or M1-BSA, and incubated as described in Methods. If the YPD culture was washed and inoculated into these media, proteinase secretion was delayed by over 24 h. The level of proteinase in CS was determined by milk-agarose spot assay. No proteinase was detected in CS of M1-AS, though *C. albicans* grew better in M1-AS until about 16 h, as compared to M1-BSA. The proteinase level in CS of M1-BSA increased exponentially until 16 h (Fig. 3), after which it remained constant for at least 24 h (data not shown); most of the proteinase was secreted between 10 to 16 h. Proteinase secretion was also checked by Western blot analysis. In spite of the greater sensitivity of this method, no proteinase was detected in CS of M1-AS (Fig. 4). The degradation seen in the last two lanes of M1-BSA culture was insignificant, since proteinase level, in terms of activity, remained constant for several more hours. Since Western blot analysis is an extremely sensitive technique, it can detect degradation products present in subnanogram amounts. When fully induced, proteinase accumulated in M1-BSA culture to about a 1000-fold higher level than in M1-AS culture, since, in a Western blot, the proteinase band was seen at 1024-fold dilution of the CS of the former, but it was not detected even in the undiluted CS of the latter (data not shown). Under a different set of inducing conditions, proteinase level in the CS initially increased rapidly, decreased later, and then increased again (Ross *et al.*, 1990). Under our conditions, the proteinase level remained constant until at least 24 h from the highest level of proteinase reached at 16 h. The variation in the pattern of induction might be due to the different inducing conditions employed.

Proteinase secretion was also studied by *in vivo* labelling and immunoprecipitation. The affinity-puri-

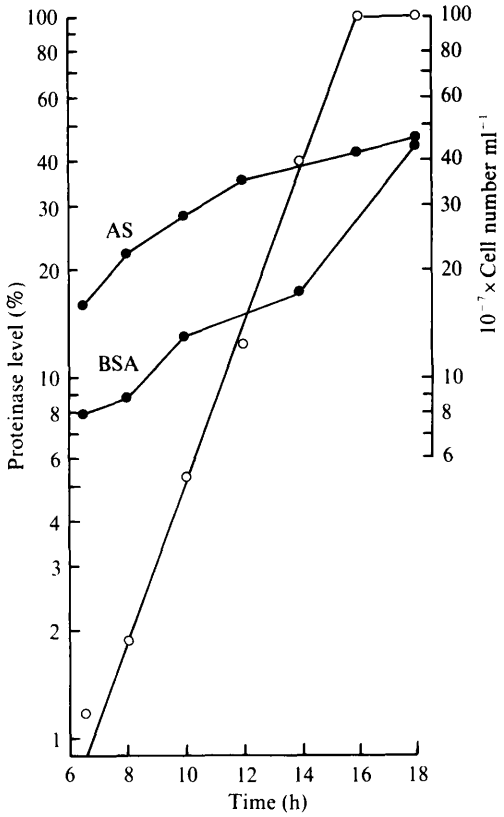

Fig. 3. Kinetics of proteinase secretion. A 14 h YPD culture of *C. albicans* strain SC5314 was diluted 100-fold in M1-AS or M1-BSA. Cell number (●) and proteinase level (○) (by milk agarose spot assay) were determined at different times. CS from M1-AS did not show any proteinase activity. The proteinase level is expressed as a percentage of the level at 18 h, after which there was no further increase.

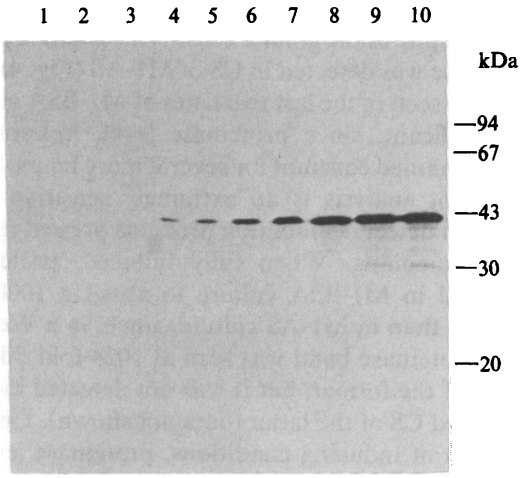

Fig. 4. Kinetics of proteinase secretion by Western blot analysis. Culture supernatant (16 µl each) of *C. albicans* strain SC5314, grown with either M1-AS (lanes 1 to 3) or M1-BSA (lanes 4 to 10), were resolved on a 10 to 17% (w/v) gradient SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified anti-proteinase antibodies. CS of M1-AS cultures was collected at 6·5 h (lane 1), 14 h (lane 2) and 18 h (lane 3), and that of M1-BSA culture at 6·5 h (lane 4), 8 h (lane 5), 10 h (lane 6), 12 h (lane 7), 14 h (lane 8), 16 h (lane 9) and 18 h (lane 10).

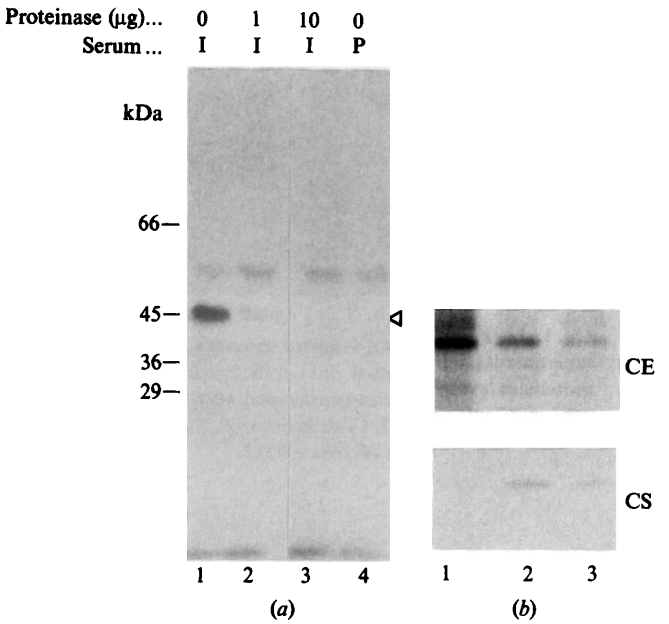

Fig. 5. Autoradiograph from *in vivo* labelling and immunoprecipitation of BSA-induced cells. (*a*). BSA-grown culture was labelled *in vivo* with 50 µCi (1·9 MBq) [^{35}S]methionine ml^{-1} for 20 min and immunoprecipitated with affinity-purified anti-proteinase antibodies (I; lanes 1 to 3) or with pre-immune serum (P; lane 4). Purified proteinase was included in two samples (lanes 2 and 3; 1 and 10 µg, respectively) during immunoprecipitation to check the specificity of the signal lighting up. The immunoprecipitates were resolved by SDS-PAGE, processed for fluorography and exposed to X-ray film. Open arrow head indicates the position of purified proteinase, which was run in a parallel lane and visualized by Coomassie Blue staining. (*b*) A BSA-grown culture was labelled *in vivo* with 100 µCi (3·7 MBq) [^{35}S]-methionine ml^{-1} for 4 min followed by addition of unlabelled methionine (200 µg ml^{-1}) for chase. Samples were removed at 0, 8 and 16 min (lanes 1, 2 and 3, respectively). Crude extract (CE) and culture supernatants (CS) were processed separately for immunoprecipitation.

fied antibodies precipitated a protein which comigrated with purified proteinase on SDS-PAGE (lane 1, Fig. 5*a*). When unlabelled purified proteinase (1 µg or 10 µg) was included during immunoprecipitation, the band was competed out. The band was not seen when pre-immune serum was used as a control (lane 4, Fig. 5*a*). These results show that the immunoprecipitated protein was proteinase. Furthermore, in a pulse chase experiment we found that the proteinase band intensity in crude cell extracts fell rapidly, while that in CS increased, showing that proteinase synthesis and secretion were coupled, and that proteinase was not accumulated in the cells (Fig. 5*b*). Ross *et al.* (1990) have also concluded, by Western blot analysis of crude extracts of induced cells, that there was no intracellular accumulation of proteinase.

Evidence for pre-proteinase

Secreted proteins are normally translated *in vivo* as larger precursors which are processed to a smaller size during

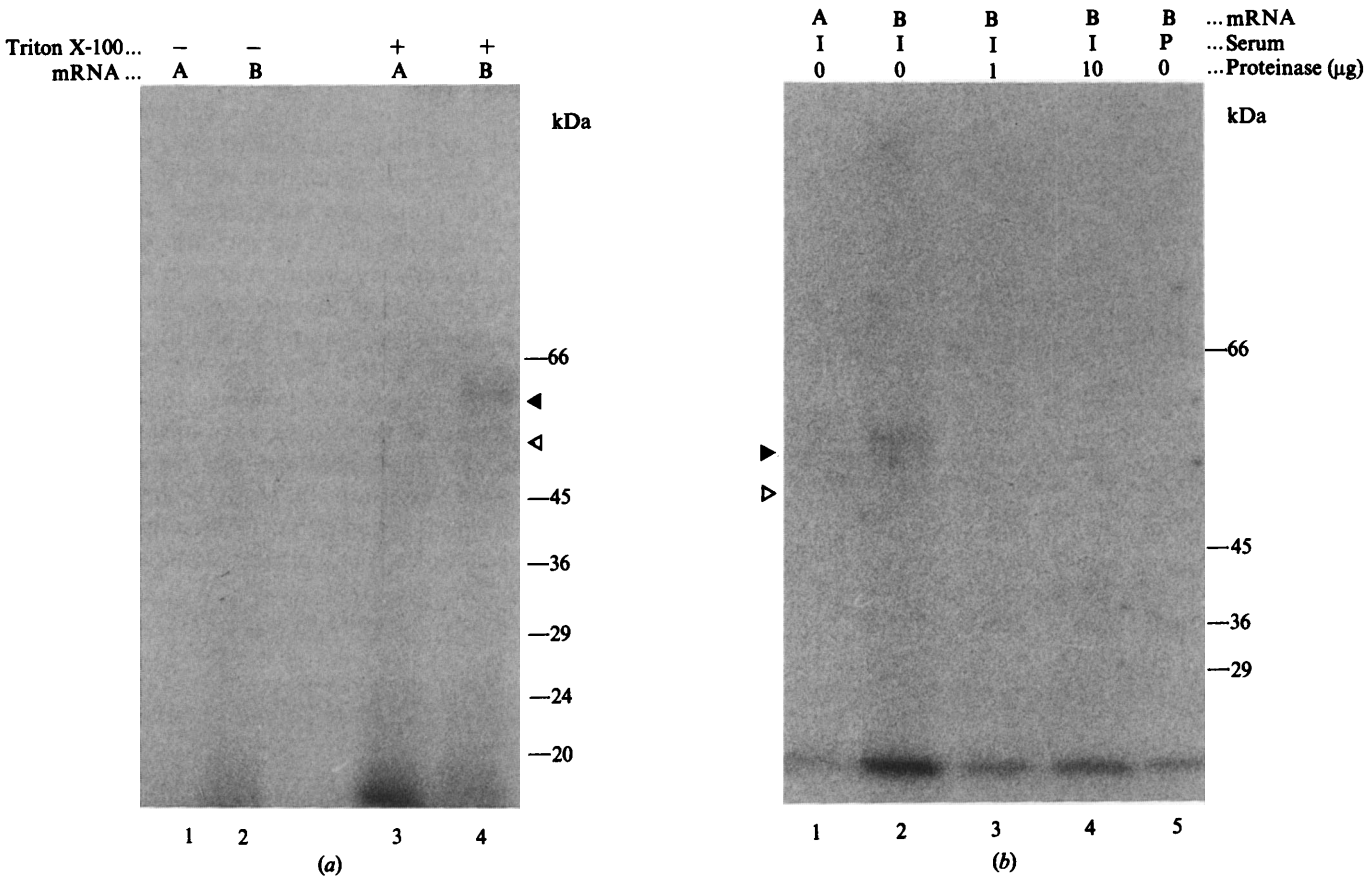

Fig. 6. Autoradiograph from immunoprecipitation of *in vitro* translated products. (*a*) mRNAs from uninduced (A, ammonium sulphate grown; lanes 1 and 3) and induced (B, BSA grown; lanes 2 and 4) cultures were translated in retic lysate with (lanes 3 and 4) or without (lanes 1 and 2) Triton X-100, immunoprecipitated with affinity-purified anti-proteinase antibodies, analysed by SDS-PAGE and processed for fluorography. Closed arrow head indicates the position of pre-protein seen in lanes 2 and 4. Open arrow head corresponds to the purified proteinase which was run in a parallel lane and visualized by Coomassie Blue staining. (*b*) mRNAs purified from uninduced (A, lane 1) and induced (B, lanes 2 to 5) cultures were translated in the presence of Triton X-100, in retic lysate, and immunoprecipitated with immune (I, lanes 1 to 4) or pre-immune (P, lane 5) serum. Purified proteinase was included in two samples (lanes 3 and 4, 1 and 10 μg, respectively) during immunoprecipitation to check the specificity of the band lighting up.

secretion (Randall *et al.*, 1987). To determine the size of the precursor form of the *C. albicans* proteinase, mRNAs isolated from induced and uninduced cells were translated in reticulocyte lysate and immunoprecipitated. The pre-proteins are not processed in the lysate system. In some experiments, Triton X-100, which stimulates translation of secreted proteins (Nash & Tate, 1984), was included. An immunoprecipitated 54 kDa band, specifically translated from BSA-induced RNA, was more intense in the presence of Triton X-100 (Fig. 6*a*). This 54 kDa band could be the precursor of proteinase. To test whether it was related to proteinase, purified unlabelled proteinase was included during immunoprecipitation as a competitor. If the 54 kDa protein was related to the proteinase, they would compete for antibody binding. Fig. 6*b* shows the disappearance of the band in lane 3 and 4, where 1 and 10 μg, respectively, of purified unlabelled proteinase were used in competition. In lane 2, no proteinase was added and a distinct band of 54 kDa was observed. Two negative controls were used. The 54 kDa band was not seen when mRNA from AS-grown cells was translated and immunoprecipitated (lane 1). In lane 5, immunoprecipitates of translation products of mRNA from BSA-grown cells with pre-immune serum were loaded. As expected, the 54 kDa band was not seen. It was therefore clear that 54 kDa protein was antigenically related to proteinase. Since the band was seen only in translation products of RNA from induced cells, it was very likely to be the pre-protein of the proteinase.

Proteinase repression by ammonium salts

Proteinase secretion was repressed when ammonium sulphate was added to either M1 or M2-BSA culture. Ammonium acetate and ammonium chloride could also repress proteinase secretion. The effect of addition of

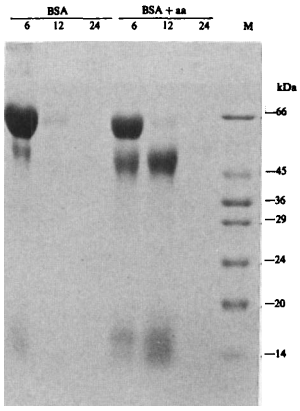

Fig. 7. Degradation of BSA during proteinase induction. CS of cultures grown with BSA or BSA and amino acid mixture (equivalent to amino acid composition of BSA) were analysed by 12% (w/v) SDS-PAGE (Coomassie Blue stain); 10 µl samples taken at 6, 12, and 24 h were analysed. M is the protein molecular mass marker (Sigma).

ammonium sulphate to a BSA culture was studied by Western blot anlaysis. Adding ammonium sulphate to a 12 h M2-BSA culture caused the proteinase level to decrease progressively, and 24 h after addition, very little proteinase was seen (data not shown). In a control M2-BSA culture, which was incubated for a similar period undisturbed, the proteinase was present at the fully induced level. When the pH of the medium was checked 24 h after addition of ammonium sulphate, it was found to be about 1·9; the pH of the medium containing only ammonium sulphate was about 1·8, and in the medium containing only BSA the pH was 4·2. Growth on ammonium sulphate appears to lower the pH of the medium, making the proteinase very unstable. Moreover, the low pH of the medium may prevent further secretion of proteinase either directly or indirectly. To find out if ammonium sulphate is directly inhibiting proteinase activity, purified proteinase was incubated

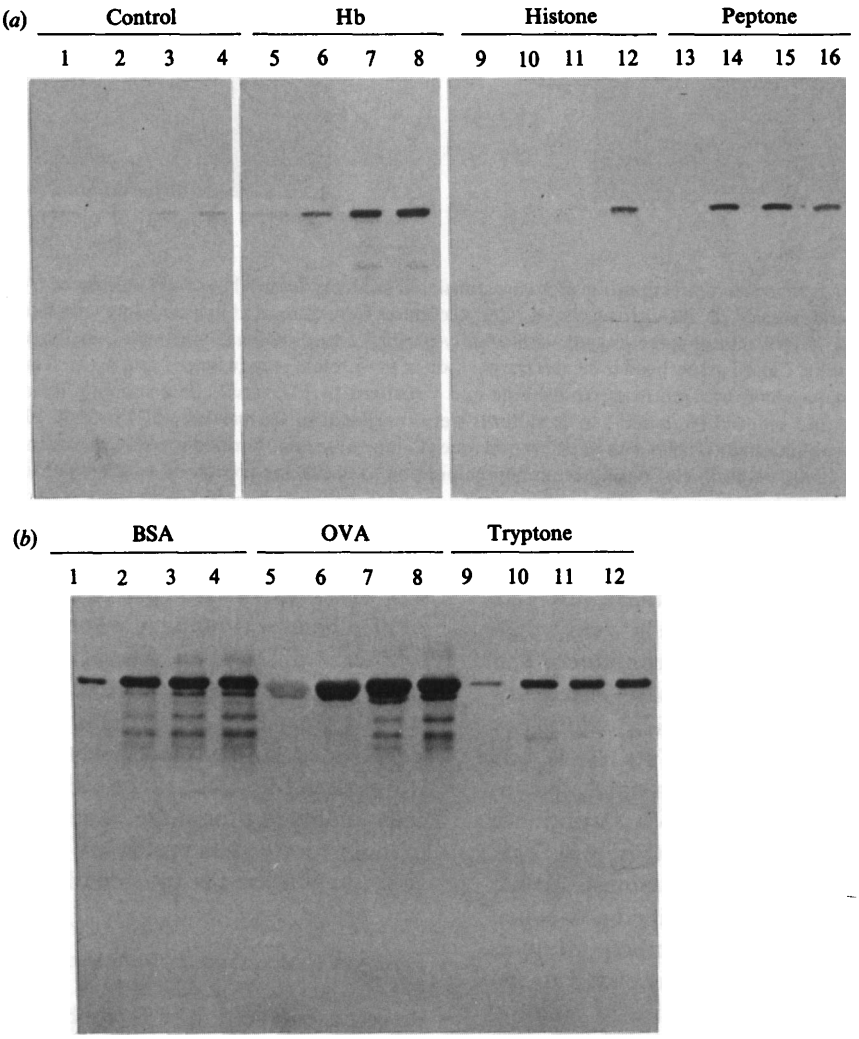

Fig. 8. Proteinase induction by various inducers. Five µl of each CS collected at 6, 12, 24 and 48 h were analysed by Western blot using affinity-purified anti-proteinase antibodies. (*a*) Lanes 1 to 4, without any inducer; lanes 5 to 8, haemoglobin; lanes 9 to 12, histone; lanes 13 to 16, peptone. (*b*) Lanes 1 to 4, BSA; lanes 5 to 8, ovalbumin; lanes 9 to 12, tryptone.

with 0·2% ammonium sulphate at room temperature for 4 h; there was no loss in activity (monitored by milk agarose assay; data not shown).

When an amino acid mixture, prepared according to the composition of BSA, was added to M2-BSA culture, both BSA degradation and proteinase induction were delayed (Fig. 7). Furthermore, when ammonium sulphate was added to M2-BSA culture, neither proteinase secretion nor BSA degradation was seen. It appears therefore that free nitrogen sources, such as ammonium salts and amino acids, repress the induction and secretion of proteinase.

Proteinase inducers

BSA and a few other proteins (e.g. ovalbumin, haemoglobin, histone and casein) were tested as substrates for proteinase in the spectrophotometric assay. Haemoglobin was the most efficiently degraded substrate (data not shown). When the same set of proteins and peptone (Difco), and tryptone (Difco) were used at 0·2%, as a nitrogen source, all could induce proteinase, but the proteinase level varied, depending on the inducer. CS samples from the induced cultures collected at different times were also analysed by Western blotting (Fig. 8). BSA, haemoglobin, and ovalbumin were efficient inducers, while histone, tryptone, and peptone were poor. Peptides present in tryptone and peptone probably serve as inducers. Casamino acids, or an amino acid mixture prepared according to the composition of BSA, did not induce proteinase (data not shown). These results suggest that proteinase is induced and secreted into the culture medium when the nitrogen source is limited to proteins and peptides, not amino acids. Further work is in progress to understand the basic mechanism of induction under nitrogen-restricted conditions.

Note added in proof. Recently, putative genes encoding secretory acid proteinase have been cloned by us (Ganesan, K., Banerjee, A. & Datta, A. 1991. *Infection and Immunity* **59**, in the Press) and by others (Hube, B., Turver, C. J., Odds, F. C., Eiffert, H., Boulnois, G. J., Kochel, H. & Ruchel, R. 1991. *Journal of Medical and Veterinary Mycology* **29**, 129–132). The sequence reported by Hube *et al.* (1991) indicates that the proteinase is synthesized as a precursor with a putative signal peptide consisting of 50 amino acids. This is consistent with our report of a preprotein for secretory acid proteinase identified by *in-vitro* translation and immunoprecipitation of RNA from BSA-induced cells.

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