2. PURIFICATION AND CHARACTERIZATION OF AN INDUCIBLE SECRETORY ACID PROTEINASE OF CANDIDA ALBICANS
2.1 SUMMARY

The acid proteinase from culture supernatants (CS) of Candida albicans was purified to apparent homogeneity by ion-exchange chromatography and chromatofocusing. Its molecular weight is approx. 45,000 and the isoelectric point is at pH 4.6. With bovine serum albumin (BSA) as a substrate, an apparent Km was $1.6 \times 10^{-4}$ M. The enzyme is inhibited by pepstatin and thus is a carboxyl proteinase. The enzyme is a glycoprotein. It clots milk at pH 5.3. At or below pH 5.0, the enzyme undergoes autodigestion. It has glutamine at N-terminus. At 60°C and above all enzyme activity is destroyed at pH 5.0.

Antibody against the purified proteinase was raised in rabbit and affinity purified. Kinetics of proteinase secretion was monitored by enzyme activity and by Western blot. With BSA as nitrogen source, proteinase is secreted at an exponential rate until about 16h of induction. Ammonium salts repress secretion of proteinase. Culture supernatant of BSA grown cultures accumulate proteinase to about a thousand fold as compared to that of ammonium sulfate (AS) grown cultures. Besides BSA, other proteins (hemoglobin, ovalbumin, histone), and peptone and tryptone when used as nitrogen source could induce proteinase. When casamino acids or an amino acid mixture (equivalent to the
composition of BSA) was used no induction was seen. When BSA along with the amino acid mixture was used induction was seen, though it was delayed.

2.2 INTRODUCTION

Candida albicans is recognized as an important pathogenic yeast causing candidosis in immunocompromised human beings and in warm blooded animals. There is an increased incidence of candidosis in the last two decades, which has been attributed to the widespread use of antibiotics and immunosuppressive agents (Odds, 1988). Among various hydrolytic enzymes reported as possible virulence factors secretory acid proteinase has gained substantial importance. Extracellular acid proteinase was first reported by Staib (1965); it has been purified and characterized by Remold et al. (1968), Macdonald and Odds (1980), Rachel (1981), and Ray and Candia (1990). Studies by Odds support the fact that proteinase is a putative virulence factor. Work by Kwon-Chung et al. (1985) Shimizu et al. (1987) also suggest the same. However, Germaine et al. (1981), Chattaway et al. (1971) throw doubt on whether this enzyme is a potential agent for pathogenicity. Germaine et al. considered that low optimum pH of the enzyme and its inhibition by salivary proteins as well as neutral pH of
saliva made it unlikely to be a significant virulence factor in pathogenesis of oral candidosis. However, vaginal fluid has a pH value which is close to the pH required for optimum proteinase activity. Furthermore besides secretion of some proteins which are its potential inducers are also seen. Also the patients suffering from vaginal candidosis secreted more proteinase compared to isolates from carriers (Cassone et al. 1987).

Proteinase can hydrolyse serum albumin and for most of the studies albumin was routinely used as a source of nitrogen for induction of this enzyme. However, Hattori et al. (1984) and Negi et al. (1984) found that C. albicans could produce a keratinolytic proteinase when grown in a medium containing human stratum corneum as a nitrogen source. The significance of proteinase in candidosis has been studied by several groups, but little detailed investigation has been done on nature of inducers and the mechanism of induction of the enzyme. In the light of these, secretory acid proteinase from C. albicans was purified and characterized. Antibody was raised against it in rabbit, and affinity purified. Using this antibody as a probe, the kinetics of proteinase secretion using various inducers (hemoglobin, histone and ovalbumin as nitrogen source) was studied. Peptone and tryptone were also used as nitrogen sources.
source for induction of proteinase. The effect of amino acids and ammonium salts on proteinase secretion was also studied.

2.3 MATERIALS AND METHODS

2.3.1 Preparation of media and solutions

a. Highest grade of reagents/chemicals available were used. For most of the experiments source of peptone was from Glaxo, India Ltd. However, occasionally peptone from Difco, USA was used, and is mentioned at the appropriate places in the text.

b. All solutions were made in double distilled water. Whenever required, solutions and media were either autoclaved at 15 pounds/Sq. inch for 20 min or were filter sterilized.

c. All % shown are on a w/v basis (unless mentioned otherwise)

d. YPD : 1% yeast extract, 2% peptone, 2% glucose.

YPD-agar : YPD broth with 2% agar

e. M1-BSA : 0.17% yeast nitrogen base without aminoacids and without ammonium sulfate (Hi media, Bombay, India), and
1% glucose autoclaved and then BSA was added to a final concentration of 0.2% from a 4% stock which was filter sterilized. A mixture of three amino acids containing L-histidine (1 mg) DL-methionine (2 mg) and DL tryptophan (2 mg) were added to one liter of medium.

f. M2-BSA : same as M1-BSA except for the mixture of three amino acids which are absent here.

2.3.2 Organism and growth conditions:

Candida albicans SC5314 was obtained from Dr. D.R. Kirsch (The Squibb Institute of Medical Research, Princeton, New Jersey, USA). It was routinely maintained on YPD-agar. A loopful of cells from fresh YPD agar slants were grown in YPD broth at 30°C with shaking for 14h (A595 reached approx. 9.0). This preculture was diluted to 100 fold into appropriate fresh broth and incubated at 30°C with shaking.

2.3.3 Enzyme assay

a) Milk agarose spot assay. The composition of milk agarose is 1% agarose, 1% skim milk and 0.114 M sodium acetate, pH 5.3, (Foltman et al., 1985). Agarose having 0.114 M sodium acetate was heated till it dissolved completely. Then 1% (v/v) milk solution was added from a
4% stock. It was mixed and poured on a glass plate that had been sealed from all sides using an adhesive tape. Culture supernatants (CS) or their dilutions, 5 μl each, were spotted, on the milk-agarose plate, through a plastic template having 5 mm diameter holes. Doubling dilutions of purified proteinase or culture supernatant 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 formed due to clotting of casein. The spot diameter of samples along with the standards were measured after 6 h to 12h incubation at room temperature. A linear relationship was obtained from a plot of diameter (in linear scale) against the amount of enzyme (in log scale), the amount of enzyme in unknown samples was calculated from the graph, as described by Schumacher et al. (1972).

b) Spectrophotometric assay. Proteinase was assayed by the method of Shimizu et al. (1987) with some modifications. The ability of the proteinase to hydrolyse BSA (at pH 3.6) was used to monitor the enzyme activity. The enzyme was incubated with 0.8% BSA in 0.05M sodium citrate buffer, pH 3.6, at 37°C for 30 min. The reaction was terminated by the addition of 5% TCA, and the precipitated albumin was removed by centrifugation in a microfuge. $A_{280}$ of supernatant was
determined. A blank tube contained all the components except that TCA was added prior to the addition of enzyme. Activity was measured as the change in $A_{280}$ nm: Experimental sample ($A_{280}$) - Control ($A_{280}$). One unit of enzyme activity is the change in one absorbance unit at 280 nm per 30 min, at 37 °C [Based on Remold et al. (1968) and Crandoll & Edwards (1987)].

2.3.4 Protein estimation:

Protein was estimated by coomassie blue dye binding method (Bradford, 1976) using bovine gamma globulin (Biorad) as standard.

2.3.5 pH and conductivity measurement:

The pH values were measured either in a Beckman pH meter or in a Globe pH meter, using pH standard buffers (Beckman). Conductivity of KCl gradient fractions was measured in a conductivity meter using KCl solution of different concentrations (0 to 1 M) as standard. KCl solutions were prepared in the same buffer as the samples. From a plot of concentration of standard solutions against its conductivity, the concentration of KCl in the samples was estimated.
2.3.6 Purification of secretory acid proteinase from *Candida albicans*:

The proteinase from culture supernatant of *C. albicans* was purified by a modified method of Shimizu *et al.* (1987).

**Step 1** A 1% inoculum from YPD 14h grown culture was transferred to M1-BSA media and grown for 48h at 30°C. Cells were pelleted at 5000 rpm for 10 min. About 850 ml of culture supernatant was collected, pH was adjusted from 4.6 to 5.0 with 100 mM trisodium citrate (pH 8.3).

**Step 2** The culture supernatant was applied at a flow rate of 40 ml per h to DE 52 column (Whatman, 1.5 by 29 cm) which had been equilibrated with 10 mM sodium citrate buffer pH 5.0 (Buffer A). The column was then washed with one bed volume of Buffer A and proteinase was eluted with a linear gradient of 0 to 1 M KCl in Buffer A (500 ml); 6 ml fractions were collected, absorbance at 280 nm was measured and salt concentrations of fractions were determined by measuring conductivity as described (Section 2.3.5).

**Step 3** The fractions with activity were pooled and dialyzed against Buffer A.

**Step 4** The dialysed fractions (36 ml) were applied at a flow rate of 15 ml per h to DEAE- Sepherose CL-6B column (Pharmacia, 1 by 15 cm) which had been equilibrated with
Buffer A. The enzyme was eluted by lowering the pH with 4 mM acidic buffer mix (4 mM each of DL-aspartic acid, L-glutamic acid and glycine in water, pH 2.6). Two ml fractions were collected. The fractions were then assayed for enzyme activity. Also, pH of all the gradient fractions were measured except that of active fractions. From a plot between fraction number and pH, the pH of the active fractions was determined. \( A_{280} \) was measured. The fractions having activity were pooled and either dialyzed against water and lyophilized or pH was raised to about 5.5 (to avoid autodigestion) and stored at 4°C or frozen.

2.3.7 Gel Electrophoresis of proteins:

a) Sodium Dodacyl Sulfate - Poly Acrylamide Gel Electrophoresis (SDS-PAGE): Electrophoresis was done using a discontinuous buffer system, essentially as described by Laemmli (1970). Gel stock solution contained 30% acrylamide and 0.8% bis-acrylamide. The concentrations of TEMED and ammonium persulfate were adjusted such that gels polymerized in about 30 min.

b)Gradient SDS-PAGE: Besides fixed concentration gels as described above, for some experiments gels having a gradient of increasing acrylamide concentration (and hence decreasing pore size) were also used.
Requirements

a) Stock acrylamide solution (30% acrylamide, 0.8% bis acrylamide)

b) Buffers
   a) 3 M Tris-Cl (pH 8.8)
   b) 1 M Tris-Cl (pH 6.8)

c) Ammonium persulfate solution (1.5%), freshly prepared

d) SDS solution (10%),
e) TEMED,
f) Gradient forming apparatus,
h) peristaltic pump,
i) magnetic stirrer

Composition of gel solution A and B are as follows:

<table>
<thead>
<tr>
<th></th>
<th>(17%) (reservoir)</th>
<th>(10%) (mixer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide-Bis (30%, 0.8%)</td>
<td>8.3 ml</td>
<td>4.9 ml</td>
</tr>
<tr>
<td>3 M Tris-Cl (pH 8.8)</td>
<td>1.88 ml</td>
<td>1.88 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.5 ml</td>
<td></td>
</tr>
<tr>
<td>APS (1.5%)</td>
<td>0.3 ml</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

and water to 15 ml in both the chambers.

Solution B was poured in the mixer, which had a tubing connected to the pump to the gel apparatus. Each solution was degassed and then added TEMED 6 μl in A and 9 μl in B. The gradient forming apparatus was used to prepare the gel.
The gel was overlayed with water. After an hour, a stacking gel of 4% acrylamide concentration was poured, Laemmli (1970).

c) Two dimensional gel electrophoresis (O'Farrell, 1975):

i) Requirements for first dimension gel solution:

Isoelectric focusing (IEF)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>6.6 g</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>1.596 ml</td>
</tr>
<tr>
<td>10% NP-40</td>
<td>2.4 ml</td>
</tr>
<tr>
<td>Deionized water</td>
<td>2.36 ml</td>
</tr>
<tr>
<td>Pharmalyte pH 2.5-5</td>
<td>600 µl</td>
</tr>
<tr>
<td>10% Ammonium persulfate(APS)</td>
<td>12 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8.4 µl</td>
</tr>
</tbody>
</table>

Deaerated this mix before adding APS and TEMED.

ii) Electrode solutions (degassed/boiled for 10 min)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Urea</td>
<td>9.5 M</td>
</tr>
<tr>
<td>NP-40</td>
<td>2%</td>
</tr>
<tr>
<td>2.5-5 Pharmalyte</td>
<td>2%</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5%</td>
</tr>
</tbody>
</table>

iii) IEF sample buffer

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>9 M</td>
</tr>
<tr>
<td>2.5-5 Pharmalyte</td>
<td>1%</td>
</tr>
</tbody>
</table>

iv) Sample overlay solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>9 M</td>
</tr>
</tbody>
</table>

v) Gel overlay solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>8 M</td>
</tr>
</tbody>
</table>
vi) **Others**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>Pierce</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>E. Merck</td>
</tr>
<tr>
<td>TEMED</td>
<td>Sigma</td>
</tr>
<tr>
<td>Alcoholic KOH solution</td>
<td>(37.6 g KOH/100 ml ethanol)</td>
</tr>
</tbody>
</table>

**First dimension: Isoelectric focusing (IEF)**

1. Glasstubes (3 mm internal diameter), were thoroughly washed with chromic acid and then placed in alcoholic-KOH solution for 12h. These tubes were rinsed with distilled water and dried. One end of the tubes was sealed with parafilm, and was marked at about 12 cm to ensure that gel lengths are equal.

2. Gel solution was poured using pasteur pipette and was overlayed with water. After polymerization, tubes were inserted into the apparatus.

3. The water from the top was replaced by 25 μl IEF sample buffer; this was overlayed with 20 μl of SOS. Upper tank was filled with 20 mM NaOH solution and lower tank with 10 mM phosphoric acid. Gels were pre run at 200 V for 15 min, 300 V for 30 min and 400 V for 30 min. After prerun, sample was loaded (Purified proteinase approx. 5 μg was lyophilized and 25 μl of IEF sample buffer was added), and gel was electrophoresed for 14h at 350v.
4. After the run, gels were carefully removed from the tubes by rimming with water filled syringe, and the acidic end of the gel was marked by making a small cut. Gels were kept in SDS-PAGE sample buffer for 30 min at room temperature, and then stored at -70°C until used for the second dimension run.

5. One of the gels was used for measuring the pH gradient; 1 cm gel slices in 4 ml degassed water kept for 10 to 12 h were used to measure pH.

Second dimension: SDS-PAGE: As described (section 2.3.7a). Standard molecular weight markers were prepared in 1% agarose blocks and placed on either side of the rod gel.

2.3.8 Biochemical properties:

Kinetic studies: With BSA as a substrate, at a concentration between 1 and 20 mg per ml, at pH 3.6, and with enzyme concentration at 170 µg per ml, activity was measured by spectrophotometric assay. Data was plotted according to Lineweaver and Burk (1934).

Effect of inhibitors: Proteinase was incubated with pepstatin (2.5 µM to 160 µM, prepared 1 mM stock in methanol) or PMSF (2.5 µM to 160 µM, 1 mM stock in
isopropanol) for 15 min at room temperature and then assayed by spotting on milk agarose plates.

**Heat stability:** Purified proteinase in 10 mM citrate buffer (pH 5.0) was preincubated for 15 min at 4, 30, 37, 45, 55, 60 and 100°C prior to the assay. Residual enzyme activity was measured at pH 3.6, spectrophotometrically.

**Linearity of the assay:** Assay was checked by using different volumes (μl) of enzyme, ranging from 2 μl to 40 μl, at pH 3.6. Another set of assay was done by varying the time of incubation, from 5 min to 1h.

**Amino acid sequence analysis:** Purified proteinase was dialyzed against water and lyophilized. N-terminal amino acid sequence was determined elsewhere independently by Dr. S. Adhya, USA and Dr. R. Nagaraj, CCMB, Hyderabad, India.

**Substrate specificity:** The suitability of hemoglobin (Hb), Ovalbumin (Ova), or histone as substrates was tested in place of BSA by the spectrophotometric assay. A 1% stock solution of each was prepared in 0.05 M citrate buffer (pH 3.6). Five μl of purified proteinase (170 μg per ml) was added in all and incubated for 10, 20 and 30 min at 37°C.
Periodic acid-Schiff staining for Glycoproteins: The procedure according to the booklet, Polyacrylamide Gel Electrophoresis, laboratory techniques, Pharmacia fine chemicals, Uppsala, 1984 was followed. The gel was first equilibrated in 7.5% acetic acid (v/v) for 45 min at 4°C, transferred to 0.2% periodic acid for 45 min at 4°C. The periodic acid was decanted and 40 ml of Schiff's reagent (gel volume was approx. 10 ml) was added, and incubated at 4°C for at least 45 min. The gel was then destained in 10% (v/v) acetic acid, at room temperature.

2.3.9 Raising of anti-proteinase antibody in rabbit and its affinity purification:

Antibody against purified proteinase was raised in a rabbit by multiple subcutaneous injections of 500 µg proteinase emulsified in Freund's complete adjuvant (Difco). After 4 weeks followed by another 2 weeks, immunization was repeated using about 250 µg proteinase emulsified with Freund's incomplete adjuvant, final shot given was intravenous. Proteinase was prepared in phosphate buffered saline (Garvey et al., 1977; Harlow and Lane, 1988). Titre of antibody was monitored by double immuno diffusion technique. Proteinase antibody was provided by my colleague Dr. K. Ganesan.
Antibody was affinity purified essentially as described by Iwaki et al. (1989). About 0.06 mg of purified proteinase was separated in a 10% SDS-PAGE, and the protein was transferred to nitrocellulose membrane. Proteinase band was visualized by Ponceau S staining as described by Salinovich et al. (1986). The band was cut out, washed in deionized water and incubated with 5% non-fat milk in TBS (50 mM Tris-Cl pH 7.6 and 0.15 M sodium chloride) for 30 min. The strip was then incubated with 1 ml of diluted antiserum (25% (v/v) in TBS) for 4h at 20°C. Then washed four times with TBS. Bound antibody was eluted in 1ml of 0.1 M glycine-Cl (pH 2.9) for 2 min in the presence of 100 μg of BSA. The eluted antibody solution was immediately neutralized with Tris-base (1M Tris, used 0.1 ml for 1 ml eluent). Affinity purified antibody was stored in TBS containing 0.1% sodium azide at 4°C.

2.3.10 Western blot analysis:

After SDS-PAGE, gel was equilibrated in Towbin's buffer (Towbin et al., 1979) containing 25 mM Tris-base, 192 mM glycine and 20% (v/v) methanol, for 30 min. A prewet nitrocellulose membrane (Schleicher and Schuell) was placed over the gel and transferred in mini electrotransfer apparatus (Hoefer, USA) for 3 h at 150 mA constant current at 15°C. After transfer, blot was removed and washed with
water and proteins were visualized by staining in Ponceau S as described (Salinovich and Montelaro, 1986) and destained in 1% (v/v) acetic acid. The positions of standard proteins were marked on the blots and dye was stripped off the proteins by washing in TBST. The filters were blocked in 5% non-fat dry milk (Amersham) in TBST (20 mM Tris pH 7.6, 150 mM NaCl and 0.05% Tween 20) for 10 to 12h at 4°C. Blots were washed thrice, for 10 min each in TBST. Primary antibody (either proteinase antiserum or affinity purified antibody at 1:1000 or 1:900 dilution respectively, in TBST) was added and incubated for 2 to 4 h at room temperature with gentle shaking. The rest of the immunodetection steps were done using Protoblot alkaline phosphatase system (Promega) or Super Screen Immunoscreening System (Amersham).

2.3.11 Kinetics of induction and its regulation:

The culture supernatants of BSA grown cultures were analysed by both activity assay as well as by Western blot at different time points. Proteins other than BSA (hemoglobin, ovalbumin, or histone), amino acids (casamino acids, 4% stock in water filter sterilized; an amino acid mixture, 2% stock in water prepared according to the composition of BSA), protein hydrolysates (peptone and tryptone)
were also checked for inducibility of proteinase at 0.2% final concentration in M-2 medium. Repression of proteinase secretion by ammonium salts was also checked similarly.

2.4 RESULTS AND DISCUSSION

2.4.1 Proteinase assay:

Proteinase level in culture supernatant was determined either spectrophotometrically or by milk agarose spot assay as described (section 2.3.3.). The milk agarose assay could detect less than 1 ng of proteinase (Fig. 2.1), and it was about 100-fold more sensitive than the other assay.

2.4.2 Purification of secretory acid proteinase:

The culture supernatant of BSA induced *Candida albicans* SC5314 yielded about 41 mg protein per 850 ml. Proteinase was desorbed from DE 52 column at about 270 mM KCl (Fig. 2.2). In the subsequent column, viz., DEAE sepharose CL 6B, the protein could be eluted with an acidic buffer mixture, pH 2.6 (Fig. 2.3). Under these conditions two isozymes were resolved which were designated as peak I and II. Pooled fractions of the first peak showed a pH of 3.4 while the second peak showed a pH of 3.2. Yield of the protein in peak II was 4.6 mg as compared to 0.19 mg in peak I. For most of the future work, peak II proteinase was
Fig. 2.1 A typical milk-agarose assay using purified proteinase. Doubling dilutions of the protein were spotted, starting from about 850 ng of proteinase, in 5 µl and incubated at room temperature (≈ 28°C) for 6 h.
Fig. 2.2 Elution profile of proteinase from the DE52 column: BSA-grown (48h) supernatant of C. albicans was applied to a column (1.5 x 29 cm) equilibrated in citrate buffer (10 mM, pH 5.0). Elution was accomplished with 500 ml of a 0 to 1 M KCl gradient in the same buffer.
Fig. 2.3 Elution profile of proteinase from DEAE-Sepharose CL-6B column: Proteinase from the DE52 column was applied to a column (1 x 15 cm) equilibrated in citrate buffer (10 mM, pH 5.0). Proteinase was eluted with a decreasing pH gradient (using 4 mM acidic buffer mixture pH 2.4, as described in Methods).
used. The culture supernatant did not have many contaminant proteins which probably could be due to the presence of proteinase. The steps involved in purification are summarised in Table 2.1. This is one of the simplest purification schemes so far reported with very high yield of the protein.

2.4.3. Purity, molecular weight and isoelectric point of proteinase:

Purity of the samples were checked by SDS-PAGE, both peak I and II showed a single band. Peak II was further analysed by SDS-PAGE using a serial dilution of the protein (Fig. 2.4A) and also a 2D-gel analysis (Fig. 2.4B). A single spot indicated the purity of the sample, the pi of proteinase of peak II according to 2-D gel was at pH 4.6. However, on chromatofocusing it eluted at a lower pH, which could be due to precipitation of the protein at its PI in the column and subsequent resolubilization and elution at a lower pH (Booklet on "Chromatofocusing with polybuffer and PBE", Pharmacia Fine Chemicals, Uppsala, 1984). From a plot of log molecular weight of standard markers versus mobility, the molecular weight of proteinase was calculated to be about 45,000.
Table 2.1
Summary of purification of *C. albicans* secretory acid proteinase

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Enzyme activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture Supernatant</td>
<td>850</td>
<td>41</td>
<td>1310</td>
<td>32</td>
<td>-</td>
</tr>
<tr>
<td>2. Pooled dialyzed DE52 fractions</td>
<td>36</td>
<td>4.8</td>
<td>752</td>
<td>157</td>
<td>57</td>
</tr>
<tr>
<td>3. DEAE-Sepharose CL-6B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Peak I</td>
<td>6</td>
<td>0.19</td>
<td>34</td>
<td>179</td>
<td>2.6</td>
</tr>
<tr>
<td>b) Peak II</td>
<td>15</td>
<td>4.6</td>
<td>493</td>
<td>107</td>
<td>38</td>
</tr>
</tbody>
</table>
Fig. 2.4 Electrophoretic analysis of purified proteinase: (A) Doubling dilutions of proteinase (lanes 1 to 6), starting with 2 µg (lane 1), was analysed by 12% SDS-PAGE. Lane M has SDS-7 (Sigma) calibration proteins for molecular weight estimation. (B) Five µg of proteinase was analysed by two-dimensional gel electrophoresis. The first dimension was IEF with pH 2-5 ampholytes (Pharmacia) and the second dimension was 12% SDS-PAGE.
2.4.4 Linearity of the spectrophotometric assay:

Purified proteinase (170 μg/ml) in citrate buffer (pH 3.6.) was incubated at various concentrations for 30 min at 37°C and was found to be linear upto 2 μg (data not shown). When the same was incubated for different time periods 5, 10, 20, 30, 45 and 60 min, linearity was maintained till 30 min.

2.4.5 Kinetics and inhibition:

Kinetic properties of Candida albicans proteinase were tested with bovine serum albumin as substrate, 1 to 20 mg per ml and with enzyme concentration of 170 μg/ml at pH 3.6. Activity was measured by spectrophotometric assay. When plotted according to Lineweaver and Burk (1934), a linear function was obtained with an apparent Km of 1.6 x 10^{-4} M (Fig. 2.5). Inhibition of proteinase activity was tested by adding different concentrations of pepstatin and PMSF to proteinase as described (section 2.3.8). More than 80% activity was lost when pepstatin concentration was 160 μM, about 75% activity was lost when pepstatin concentration was 80 μM and enzyme concentration was 170 μg/ml. (Fig. 2.6). There was no inhibition of enzyme activity with 160 μM PMSF under similar conditions (data not shown). Since pepstatin is a specific inhibitor of carboxyl proteinases (Barrett 1980), this enzyme is a carboxyl proteinase.
Fig. 2.5 Effect of BSA concentration on the rate of proteinase activity: The data is plotted in the form of Lineweaver-Burk plot. The other assay conditions were same as described in the spectrophotometric assay. Substrate (BSA) concentration is expressed in percent.
Fig. 2.6 Inhibition of proteinase by pepstatin: Five µl of purified proteinase (170 µg/ml) was pre-incubated (15 min) with 5 µl of different concentrations of pepstatin (0-160 µM) at room temperature. Five µl of each was spotted on milk agarose plate and incubated for 9 h at room temperature. Doubling dilutions of purified proteinase was also spotted as control; activity (%) of the samples remaining after treatment was calculated from the spot diameter of the control (taking the largest diameter as 100 % activity). Result showed that at 80 µM pepstatin about 25 % of the enzyme activity remained.
2.4.6 Thermal stability:

Purified proteinase (in 10 mM citrate buffer, pH 5.0) preincubated at various temperatures as described (section 2.3.8) revealed that till 45°C the enzyme exhibited 100% activity as compared to the control sample incubated at 37°C. At 55°C, about 60% of the activity was retained, but at 60°C and above no activity could be detected.

2.4.7 N-terminal amino acid sequence

The N-terminal sequence is

GLN-ALA-VAL-PRO-VAL-THR-LEU

N-terminal amino acid of Candida albicans proteinase as reported by Ruchel (1981) is tryptophan.

2.4.8. Glycoprotein staining:

The Candida albicans proteinase is a glycoprotein as evidenced by staining with PAS (Section 2.3.8). Purified proteinase (about 5 μg) showed a dark pink band (data not shown). Proteinase undergoes autodigestion at and below pH 5.0 (Ruchel,1981). Most of the degraded fragments also stained for glycoprotein. All the properties of proteinase studied here are summarised in Table 2.2.
Table 2.2

Biochemical properties of secretory acid proteinase from C. albicans

<table>
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<tr>
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<td>4</td>
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<td>a</td>
<td>Pepstatin</td>
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<td>b</td>
<td>PMSF</td>
<td>No effect</td>
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<td>7</td>
<td>Km with BSA as substrate</td>
<td>$1.6 \times 10^{-4}$ M</td>
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2.4.9. Proteinase secretion by *Candida albicans*.

When YPD preculture was used, proteinase level in culture supernatant of M1-BSA increased exponentially up to 16h. Cell number was calculated from $O.D_{595}$ vs cell number, which was determined from haemocytometer counts. Buds were also counted as individual cells.

*C. albicans* grew better in M1-AS until about 16 h though no proteinase was secreted (Fig. 2.7A). The rate of proteinase secretion as percent of the final level was calculated. Fig. 2.7B shows that proteinase secretion stops after 16 h; between 10 to 16h most of the proteinase is secreted out. Proteinase secretion was also checked by Western blot analysis (Fig. 2.8A). Inspite of the increased sensitivity, no proteinase was detectable in culture supernatant of M1-AS. When 14h grown YPD was used as a preculture for M1-BSA media, from about 6.5h onwards proteinase could be detected by Western blot analysis (Fig. 2.8A). Proteinase when fully induced accumulates in M1-BSA to about 1000 fold higher level compared to M1-AS culture (Fig. 2.8B). When *C. albicans* was inoculated in YPD (peptone from Glaxo India Ltd) proteinase activity could be detected even in culture supernatant of YPD culture, by milk agarose as well as by Western blot. One of the reasons for earlier appearance (from about 6.5 h) of proteinase (in M1-BSA) could be due to
Fig. 2.7 Kinetics of proteinase secretion:
(A) Proteinase level in CS: *C. albicans* grown in YPD for 14 h was diluted 100-fold in M1 containing AS or BSA as a nitrogen source. Cell number and proteinase level were determined at different time intervals. The proteinase level was determined by milk agarose spot assay. CS of M1-AS did not show any proteinase activity. The proteinase level in M1-BSA culture is expressed as % of the level at 18 h, after which there was no further increase.
(B) Rate of proteinase secretion: Proteinase secreted every 2 h, as % of the final level, was calculated from data of (A), assuming that there is no turnover of proteinase in CS.
Fig. 2.8 (A) Kinetics of proteinase secretion (Western blot analysis): CS (16 μl each) of *C. albicans* grown with either AS (lanes 1 to 3) or BSA (lanes 4 to 10) for 6.5 h (lanes 1 and 4), 8 h (lane 5), 10 h (lane 6), 12h (lane 7), 14 h (lanes 2 and 8), 16 h (lane 9) and 18 h (lanes 3 and 10), were resolved on 10 to 17% gradient SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified antiproteinase antibody.

(B) Proteinase level in CS: Western blot analysis of 18 h cultures of *C. albicans* grown with BSA or AS. Undiluted CS (16 μl each) or doubling dilutions of BSA-CS (16 μl each) were resolved on a 10 to 17% gradient SDS-PAGE, transferred to nitrocellulose and probed with affinity-purified antiproteinase antibody. In the original blot, the proteinase band was seen even at 1024-fold dilution, and none in the AS lane.
secretion of proteinase in YPD preculture, which in turn degrades BSA for further induction. This view is strengthened by the results where YPD preculture was washed twice in water and transferred to M1-BSA media to devoid any existing proteinase, till 24h no proteinase could be seen. Furthermore the source of the chemicals are important; as observed when preculture was grown in YPD (Difco peptone), there was no induction of proteinase. Appearance of proteinase in both M1 and M2 media without any nitrogen source was detected by Western blot, however level of proteinase secreted was very low.

2.4.10 Proteinase repression by ammonium sulfate.

Proteinase secretion is repressed when ammonium sulfate is added to either M1-BSA or M2-BSA culture. Ammonium acetate and ammonium chloride could also repress proteinase (data not shown). Fig. 2.9 shows the effect of ammonium sulfate on proteinase secretion; when ammonium sulfate was added to 12h M2-BSA culture, proteinase level in the culture supernatant progressively decreased and by 24 h of ammonium sulfate addition (lane 10), very little proteinase could be detected. However, when pH of the medium was checked after 24 h of ammonium sulfate addition (by the time proteinase had diminished), it was found to be about 1.9; the pH of the medium containing only ammonium sulfate as nitrogen source.
Fig. 2.9 Western blot showing the effect on proteinase level of the addition of AS to a 12 h M2-BSA culture: Five μl of CS were analysed. Lane 1, 24 h BSA culture; lane 2, 24 h AS culture; lanes 3 to 10, samples were taken at different times after AS addition: lanes 3, 0 h; 4, 1 h; 5, 3 h; 6, 5 h; 7, 7 h; 8, 10 h; 9, 12 h; 10, 24 h after addition of AS.
was about 1.8 and in the inducing medium containing BSA instead of ammonium sulfate, the pH was 4.2. Since growth on ammonium sulfate appears to lower the pH of the medium, the mode of control by ammonium sulfate could be as follows: the disappearance of the existing proteinase could be due to instability of the enzyme at low pH (about 1.9), moreover the low pH of the medium could, by a cascade of changes, prevent further secretion of proteinase. Western blot analysis demonstrates that in BSA grown culture supernatants proteinase is stable at least for 24 h. To find out if ammonium sulfate is directly inhibiting proteinase activity, purified proteinase was incubated with 0.2% ammonium sulfate at room temperature for 4 h; activity was monitored by milk agarose assay which showed that there was no loss in activity.

Induction of proteinase: a probable mechanism.

While studying the kinetics of proteinase secretion using BSA as a nitrogen source, a characteristic degradation pattern of BSA could be seen in the blot by Ponceau S staining. By 12 h almost no BSA was visible. When ammonium sulfate or other ammonium ions were present along with BSA from the very beginning, no degradation of BSA was seen. This apparently suggests that the proteinase which is secreted out acts on BSA and degrades it to peptides and aminoacids.
which in turn induces further secretion of proteinase. From the above observations, it appears that degradation of the inducer could be correlated to the secretion of proteinase. Lack of free source of nitrogen i.e. when the nitrogen source is limited to proteins and peptides, proteinase is induced and secreted out in the medium which in turn degrades the available protein. Partially degraded protein in turn induces further secretion. When casamino acids or a mixture of amino acids (equivalent to the composition of BSA) was used as a nitrogen source, no proteinase induction was seen. Furthermore when an amino acid mixture was added to the M2-BSA, both induction of proteinase and degradation of BSA were delayed (Fig. 2.10). Ammonium sulfate acts as a repressor of proteinase induction whereas aminoacid mixture is not. It is utilized in preference to BSA, thereby delaying the induction of proteinase.

2.4.11 Substrate specificity of proteinase:

A few other proteins were tested as substrates for proteinase. Hemoglobin was the most efficiently degraded substrate (Fig. 2.11 A). When same set of proteins, peptone (Difco) and tryptone (Difco) (0.2% each) were used as nitrogen source, all of them could induce proteinase (Fig. 2.11 B), but proteinase level varied depending on the nature of inducer. Culture supernatants of above samples
Fig. 2.10 Degradation of BSA during induction: CS grown in BSA, or BSA and amino acid mixture (equivalent to amino acid composition of BSA) were analysed by 12% SDS-PAGE (Coomassie blue stain) taking 10 μl samples at 6, 12, and 24 h. M is the molecular mass marker proteins (Sigma).
Fig. 2.11 (A). Substrate specificity of proteinase: Proteinase was assayed spectrophotometrically. It was incubated with different substrates (hemoglobin, histone, ovalbumin and BSA), at 37°C for 10 to 30 min.

(B) Proteinase induction by various proteins, peptone and tryptone: At 6, 12, 24, and 48 h samples were collected and 5 µl of each CS was spotted on milk agarose plates. Proteinase level is expressed as % of that in the 48 h BSA culture.
were collected at 6, 12, 24, and 48 h and analysed by Western blot (Fig. 2.12 A, and B). While BSA, hemoglobin and ovalbumin are efficient inducers, histone, tryptone and peptone are not. It appears that the peptides present in tryptone and peptone probably serve as inducers. With ovalbumin as inducer, proteinase appeared to be more and bands looked diffused, which could be due to co-migration of proteinase and ovalbumin.

In conclusion, results show that 1. the time course of proteinase secretion depends on the nature of preculture used. 2. With BSA as nitrogen source proteinase is secreted out at an exponentially increasing rate until about 16 h of induction. 3. Culture supernatant of BSA grown cultures accumulate proteinase to about a thousand fold higher level compared to that of ammonium sulfate grown cultures. 4. Besides BSA, other proteins, and peptone and tryptone when used as nitrogen source, could induce proteinase. 5. Amino acid mixture as nitrogen source, could not induce proteinase. 6. Ammonium sulfate represses the secretion of proteinase and the rate of turn over in the culture supernatant is also apparently increased.

Regulation of proteinase expression can be studied in detail using cloned gene (refer chapter 3).
Fig. 2.12 Proteinase induction by various inducers in M2 medium: Western blot analysis using affinity-purified antiproteinase antibody. Five μl each of CS collected at 6, 12, 24, and 48 h were analysed.

(A) Lanes 1 to 4, without any inducer; lanes 5 to 8, hemoglobin; 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.

The blots shown in panel A and B were processed at different times and hence the variation in the signal intensities.