Volume 327, number 1, 108–112 FEBS 12741 July 1993


# *Candida parapsilosis* expresses and secretes two aspartic proteinases

Martin Fusek[a], Elizabeth A. Smith[a], Michel Monod[b] and Stephen I. Foundling[a]

[a]*Laboratory of Protein Crystallography, Protein Studies Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA* and [b]*Laboratoire de Mycologie, Service de Dermatologie, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland*

Received 27 May 1993

We have isolated and characterized a second aspartic proteinase secreted by the CHUV E-18 strain of *Candida parapsilosis*. This proteinase is produced at a level corresponding to approximately 25% of the production of the main proteinase described earlier [1]. This minor proteinase has similar molecular weight and pH optimum but differs in the isoelectric point and in the specificity when compared with the major secreted form. The determination of the amino terminal amino acid sequence identified this minor form of *Candida parapsilosis* aspartic proteinase as a protein which corresponds to the sequence deduced from genomic DNA originally reported as a pseudogene [1]. We conclude that strain CHUV E-18 of *Candida parapsilosis* expresses and secretes two different aspartic proteinases.

Aspartic proteinase; Proteinase inhibitor; Pseudogene, *Candida parapsilosis*

## 1. INTRODUCTION

Infections caused by *Candida* opportunistic yeasts are becoming one of the major threats for immune compromised patients [2], such as those infected with human immunodeficiency virus, cancer patients, transplant recipients and patients with other immunodeficiencies. The conversion of the otherwise nonvirulent and mildly pathogenic *Candida* microorganism to an invasive one is being connected to several secreted virulence factors [3,4]. Among these factors are the extracellular aspartic proteinases. These enzymes are considered to be the contributing part of the mechanism by which *Candida* penetrates the host's mucosal membranes [5]. Several of the extracellular aspartic proteinases were isolated and partially characterized from the yeasts *Candida albicans* [6], *Candida tropicalis* and *Candida parapsilosis* [7,8]. The amino acid sequences of these aspartic proteinases were deduced from the corresponding genomic DNA sequences [1,9,10].

Based upon the invasive role that *Candida* aspartic proteinases are suggested to play in the development of severe systemic *Candida* infection and disseminated candidamycoses (recently summarized by Rüchel et al. [5]), these proteinases offer a new target for the synthetic inhibitor design.

A wealth of information exists for the inhibition of several other aspartic proteinases which has been gained during the design of inhibitors for human renin [11], and for the retroviral proteinases of the AIDS virus [12]. These studies show that substrate specificity in the aspartic proteinase family is complex and composite. Chemical specificity is essentially the sum of the numerous interactions of amino acid side chains, peptide backbone atoms and substrate atoms in combination, interacting in total in the active site cleft of the enzyme. Even minor differences in the primary and hence the tertiary structures of these enzymes can lead to markedly different substrate specificities.

It was shown recently that there is a difference in primary structures of the secreted aspartic proteinases from different strains of *Candida albicans* yeast [5]. These differences in primary structure affect both the chemical properties and specificity of these enzymes [5]. When examining the sequences of the genomic DNA from *Candida parapsilosis* coding for aspartic proteinases, Veragh et al. [1] have detected two different DNA sequences coding for putative extracellular aspartic proteinases. One of the genes was identified by amino terminal sequencing of the extracellular protein as the secreted aspartic proteinase, but the second gene, since its product was not detected, was considered an untranslated gene or pseudogene.

In this paper we report new data which shows that the product of the second aspartic proteinase gene is found in *Candida parapsilosis* cultures grown in liquid media with BSA as the sole source of nitrogen. This proteinase

*Correspondence address* S. Foundling, Laboratory of Protein Crystallography, Protein Studies Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA
Fax: (1) (405) 271 3980

*Abbreviations*: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; CPAP, *Candida parapsilosis* aspartic proteinase


*Published by Elsevier Science Publishers B.V*

is secreted and has very similar molecular weight but different isoelectric point and catalytic properties compared to the major secreted enzyme.

## 2. EXPERIMENTAL

### 2.1. *Yeast culture*

*Candida parapsilosis* CHUV E-18 is maintained and replated every 6 to 8 weeks on Sabouraud agar plates (Difco). For growing *Candida* yeast in liquid culture the following procedure is used. 50 ml of sterile 1 2% YCB medium (Difco) containing 0.2% of bovine serum albumin (BSA) (Sigma, USA) is adjusted to pH 4.0 and inoculated with several colonies of *Candida* yeast from agar plates. This culture is grown overnight at 30°C in an orbital incubator shaker (200 rpm). The next morning 0.5 ml of this overnight culture is used for the inoculation of 500 ml of the same culture media. After 4 days of growth at 30°C, the 500 ml cultures are centrifuged (30 min at 7,000 × *g*) and the supernatant is sterile filtered to remove any infectious cellular material. This sterile supernatant is used for enzyme isolation.

### 2.2. *Isolation of aspartic extracellular proteinases*

20 ml of SP-Trisacryl resin (IBF, France) is added to the sterile filtered supernatant for batch absorption of the enzyme (the resin is pre-equilibrated in 15 mM sodium citrate, pH 4 0). Before the addition of the ion exchange resin the pH of the supernatant is adjusted to pH 4 0. The resin is removed after 30 min of stirring at room temperature, washed thoroughly with 15 mM citrate buffer pH 4.0 and then the proteinase is eluted from the resin using 300 mM citrate buffer pH 4.0. Eluted proteins are then dialyzed against the washing buffer overnight and applied to a Mono-S cation exchange FPLC column (5 × 5, Pharmacia, Sweden). This step provides the final purification step and elution from the column is effected using a linear gradient of NaCl as shown on Fig. 1. The yield of the two proteins defined here as CPAP#1 and CPAP#2 are 4 and 1 mg/l of culture, respectively (CPAP, *Candida parapsilosis* Aspartic Proteinase).

### 2.3. *SDS electrophoresis, isoelectric focusing and Western blotting*

The SDS polyacrylamide electrophoresis is carried out using a 4% stacking gel pH 6.8 and 15% separating gel pH 8.8 with 0.1% of SDS in all buffers. Samples were reduced with $\beta$-mercaptoethenol before application on the gel. Isoelectric focusing is carried out using a Pharmacia Phast System (Pharmacia, Sweden) using precast Pharmacia IEF gels (pI 3–9). Western blotting is performed on a semi-dry electroblotter (Enprotech, USA) using the standard procedures recommended by the manufacturer. Antibodies used for western blotting were as described by Togni et al. [10].

### 2.4. *Activity determination*

The proteolytic activity in chromatographed fractions is determined using two photometric methods. For fast screening of activity in eluted fractions we use a solution of 1% bovine hemoglobin (Sigma, USA) dissolved in 100 mM sodium citrate buffer, pH 3.4 Aliquots (5 $\mu$l) of the eluted fractions are incubated with 1 ml of the hemoglobin solution at 37°C for 30 min. The reaction is stopped by the addition of 200 $\mu$l of 10% trichloracetic acid. Any precipitate is removed by centrifugation and the absorbance of the supernatant is read at 280 nm. This photometric absorbance assay is linear in the range of 0.01 to 1.0 AU. One unit of specific activity is defined as the change in absorbance at $A_{280nm}$ of 1 AU by 1 mg of proteinase in incubation time of $T$ = 30 min.

The kinetic measurements were made using a spectrophotometric synthetic peptide substrate Lys-Pro-Ala-Glu-Phe-Phe($p$-$NO_2$)-Ala-Leu, where Phe($p$-$NO_2$) is the *para*-nitrophenylalanyl group. The cleavage of the bond between the Phe and Phe($p$-$NO_2$) residues yields a decrease in absorbance at 310 nm. Measurements of initial rates of this change are measured to give initial velocity [13]. Measurements are done in 100 mM sodium citrate pH 3.5 monitoring the change in absorbance at 310 nm using a Varian DMS model 6000 spectrophotometer (Varian, Austria).

### 2.5. *Amino terminal sequencing*

Amino terminal sequencing was done on an ABI gas sequencer using the procedures and chemicals recommended by the manufacturer.

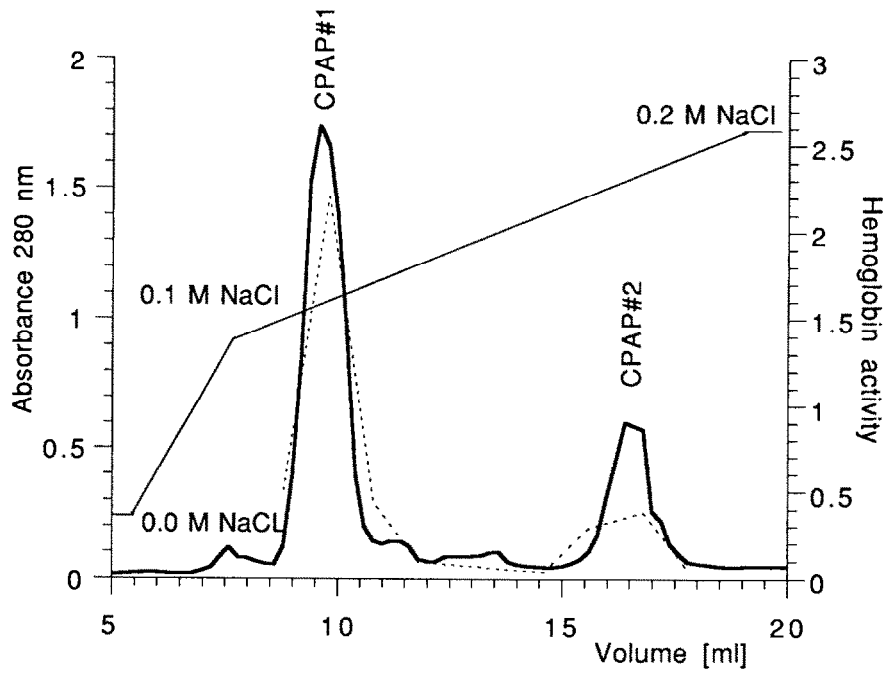


Fig. 1. A chromatogram of the isolation of two aspartic proteinases from the supernatant of liquid culture of *Candida parapsilosis* yeast. The column used was a Pharmacia Mono S (5 × 5), equilibrated in 15 mM citrate pH 4.0 buffer. Flow rate was set at 1 ml·min$^{-1}$. The NaCl elution gradient developed is shown in the diagram. The solid line plot shows the absorbance profile for the eluted CPAP#1 and CPAP#2 and the dashed line overlaid shows the correlated proteolytic activity for the two proteinases.

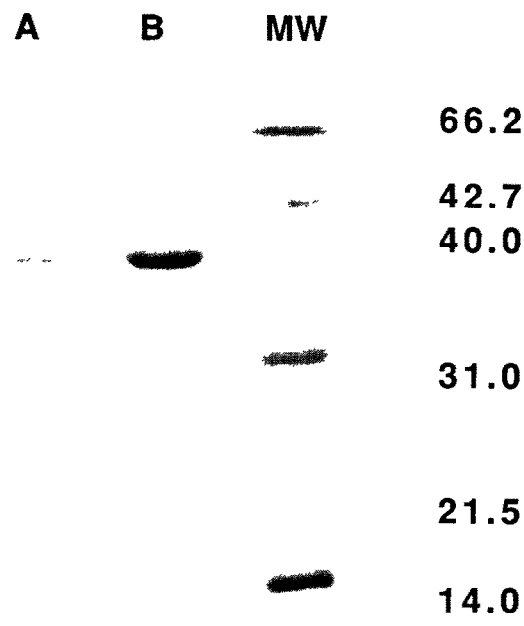


Fig. 2. Analysis of the molecular weights of the purified *Candida parapsilosis* aspartic proteinases by Coomassie blue (R250) stained 15% SDS-PAGE gel. Both CPAP#1 (lane A) and CPAP#2 run with at similar positions and with estimated molecular weights in the range of 35 kDa to 38 kDa.

## 3. RESULTS AND DISCUSSION

During the isolation of CPAP using the Mono S column, we observed a second distinct peak which eluted later in the NaCl gradient (Fig. 1). This second minor peak had proteolytic activity at pH 3.4 and it was labelled as CPAP#2. The protein was first compared with the CPAP#1 by SDS electrophoresis (Fig. 2), then by western blot analysis using antibodies raised against CPAP#1 [14] (data not shown) and finally by isoelectrofocusing gel electrophoresis (data not shown). The electrophoretic mobility was the same for both proteins and CPAP#2 crossreacted with antibodies to CPAP#1. The isoelectric point for CPAP#1 was found to be 5.3 and 6.4 for CPAP#2. This difference in isoelectric point explains the difference in the retention on the Mono S column of CPAP#2. These data are in good agreement with the data by Rüchel [14] who observed a minor impurity with the pI 6.5.

When comparing the activity of two proteinases using macromolecular substrates (hemoglobin and BSA) we found that the specific activity of the minor peak is similar (Fig. 3 and Table I) and the products of the cleavage of BSA gave a similar pattern for both enzymes (Fig. 3). Both enzymes had an apparent pH optimum close to pH 3.5 (Fig. 2) and both were inhibited by pepstatin A.

A large difference was observed between CPAP#1 and CPAP#2 when the rates of cleavage of the synthetic spectrophotometric substrate were measured. The difference in initial rates of catalytic activity was more than 2 orders of magnitude between CPAP#1 and CPAP#2. CPAP#2 has a lower activity towards this synthetic substrate (Table I). This indicates that the two proteinase species CPAP#1 and CPAP#2 have a different chemical structure, particularly in the region of the substrate binding site which is responsible for substrate recognition and specificity of binding.

In order to determine the molecular differences between the two proteins we determined the amino terminal amino acid sequences. As seen in Fig. 4, the sequence of CPAP#1 corresponds to the reported amino terminus of the genomic gene sequence for the major aspartic proteinase. The second peak CPAP#2, is identified as the protein derived from the putative untranslated genomic gene sequence, or pseudogene.

The authors in the original paper [1] have demonstrated that there are two different gene sequences present in the strain CHUV E-18 of *Candida parapsilosis*,

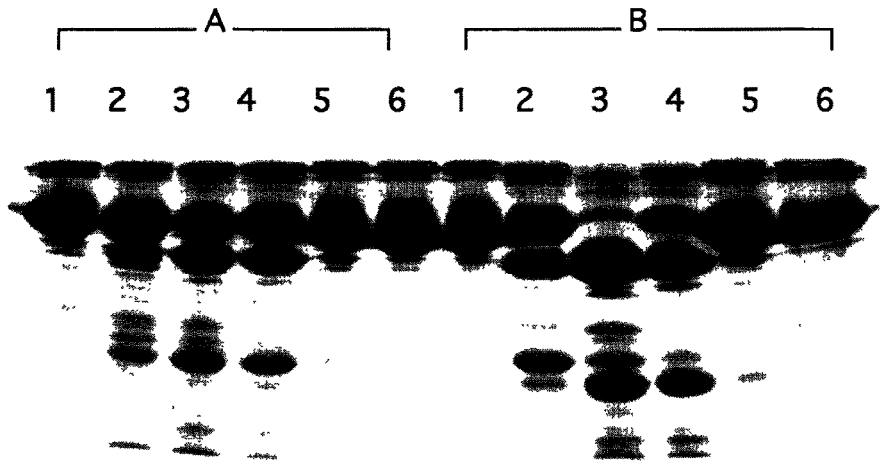


Fig. 3. pH profile of cleavage of BSA by the two aspartic proteinase from *Candida parapsilosis*. Panel A, CPAP#1; panel B, CPAP#2. Lane 1, pH 2.5; lane 2, pH 3.0; lane 3, pH 3.5; lane 4, pH 4.0; lane 5, pH 4.5; and lane 6, pH 5.0.

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          1   .   10    .   20    .   30    .   40    .   50
CPAP#1 MVAIVTLTRQVLLTIALALFAQGAAIPEEAAKRDDNPGFVALDFDVLRKP
                                        **
CPAP#2 MTTIAIFTKNVLLAIAFALFAQGAAIPD-PAKRDDNPGFVALDFEVTRKP

                .   60    .   70    .   80    .   90    .  100
CPAP#1 LNLTEALLREKRDSISLSLINEGPSYASKVSVGSNKQQQTVIIDTGSSDF
                          **                    ^^^
CPAP#2 LDVNATSELSKRSSPSSPLYFEGPSYGIRVSVGSNKQEQQVVLDTGSSDF
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Fig 4 Sequences of two *Candida parapsilosis* aspartic proteinases [1]. The amino acids underlined correspond to the sequences determined by N-terminal sequencing of the isolated enzymes CPAP#1 and CPAP#2. Key: CPAP#1, the deduced amino acid sequence for the first 100 residues of *Candida parapsilosis*, the major isolated form of the aspartic proteinase; CPAP#2; the partial amino acid sequence of *Candida parapsilosis*, the minor isolated form of the aspartic proteinase. The amino acid sequence is given in the single residue code. Characters defined in **bold** type are those residues that are absolutely conserved between the two enzymes. Two **Lys-Arg** peptidase processing sites are marked with an asterisk. The characteristic catalytic motif of the aspartic proteinase family of enzymes, **DTG**, is indicated by up arrows (**D**, catalytic aspartic acid; and residues of the firemen's grip, **TG**). The experimentally defined amino terminal sequences of the two secreted enzymes are underlined.

and two experiments were used to identify the secreted products of these two genes. Primarily only one of the gene products (CPAP#1) was identified by sequencing of isolated protein from yeast culture supernatant. Secondly a mutant of *Candida tropicalis*, whose ability to express an active proteinase had been disrupted was transformed with either of the *parapsilosis* genes. Restoration of proteolytic activity were only observed in the transfection with the CPAP#1 gene. The conclusions from these experiments strongly suggested that the CPAP#2 was either a pseudogene or its product was expressed under as yet unidentified physiologic conditions.

Our experimental data shows that the product of the CPAP#2 gene, the CPAP#2 aspartic proteinase is expressed and secreted. The behavior of the CPAP#2 proteinase during the gel filtration purification step will be similar as the CPAP#1 proteinase. This protein also constitutes only about 20% of the total enzyme recovered from the culture supernatant. These facts combined with a specific amino terminal sequence formed by Ser-Ser-Pro-Ser-Ser-Pro that gives a very low signal during amino terminal sequencing are probably responsible for the original observations that the product of the CPAP#2 gene went undetected. Why the complementation of the proteinase deletion mutant of *Candida tropicalis* by the *Candida parapsilosis* gene coding for CPAP#2 proteinase did not restore the proteolytic activity is difficult to answer. One possibility is that the CPAP#2 needs another aspartic proteinase or specific activation protease for its processing and since in the aspartic proteinase deficient mutant there was neither the original or the complementary protease, the CPAP#2 product was retained as the inactivated proenzyme.

Table I

Comparison of the proteolytic activity of two aspartic proteinase from *Candida parapsilosis*

| Enzyme | Hemoglobin ($U \cdot 10^3$) | Synthetic substrate | | |
|---|---|---|---|---|
| | | $k_{cat}$ ($s^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ ($s^{-1}\,\mu M$) |
| CPAP#1 | 145 | 36.0 | 101 | 0.3500 |
| CPAP#2 | 108 | 0.26 | 180 | 0.0014 |

Our data finally suggests that within this family of secreted *Candida* aspartic proteinases, each proteinase may show a variation in substrate specificity. This feature is not new to this family of aspartic proteinases. Several homologous structures show that all the members of this proteinase family have an extended active site cleft which can accommodate at least seven amino acids of substrate in the $S_4$–$S_{3'}$ specificity subsites [15]. The spatial arrangement of the amino acids that make up the binding cleft and subsites are responsible for the discriminatory substrate specificity of each enzyme. Very subtle changes in the enzymes amino acid sequence can lead to large differences in the substrates recognized by these enzymes.

It will be essential to map this specificity through substrate cleavage mapping for each of the *Candida* aspartic proteinases. This specificity typification is ongoing in our laboratory.

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