ENZYMOLOGICAL CHARACTERIZATION OF SECRETED PROTEINASES FROM *Candida parapsilosis* AND *Candida lusitaniae*

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Opportunistic pathogens of the genus *Candida* produce secreted aspartic proteinases (Saps) that are considered as one of the virulence factors. While Saps of *C. albicans* and *C. tropicalis* have been studied extensively, information concerning proteinases secreted by other pathogenic *Candida* species is scarce. To our knowledge, enzymologic characterizations of Sap of *C. parapsilosis* (Sapp1p) and *C. lusitaniae* (Saplp) are limited. We have purified Saps of *C. parapsilosis* and *C. lusitaniae* from yeast culture supernatants and detected only one gene product of both Sapp1p and Saplp using isoelectric focusing and N-terminal sequencing. Molecular weight of Saplp determined by SDS-PAGE is approximately 42 000. For the highest enzyme activity, both Sapp1p and Saplp require pH ranging from 3 to 4 and temperature between 27–40 °C for Saplp and 45 °C for Sapp1p, respectively. At physiological temperature, both the proteinases are stable. The characterization of Saps of clinical isolates of *C. parapsilosis* and *C. lusitaniae* should contribute to design of novel drugs specifically targeted on proteinases.

Keywords: *Candida parapsilosis*; *Candida lusitaniae*; Aspartic proteinase; pH optimum; Thermal stability; Proteolytic enzymes; Isolation; Yeasts; Anticandidal agents.

Opportunistic pathogens of the genus *Candida* cause infections representing a major threat to long-term survival of immunocompromised patients. Incidence of these pathogens has greatly increased with the introduction of broad-spectrum antibiotics, antitumor and immunosuppressive agents, as well as an increasing number of AIDS patients.

Virulence of the *Candida* pathogens is enhanced by the ability to adhere to the host surface and to penetrate to the host tissues, by the ability to undergo a phenotypic switch from yeast to hyphal form, and by production of

extracellular proteolytic enzymes[1]. In *C. albicans*, which is the most extensively studied representative of the genus *Candida*, a correlation between the virulence and secretion of aspartic proteinases has been clearly documented[2–5]. These enzymes are therefore studied as potential virulence factors and possible targets for therapeutic drug design.

Aspartic proteinases comprise a group of enzymes accomplishing various functions in viruses, bacteria, fungi, plants, and vertebrates. Secreted aspartic proteinases (Saps) produced by pathogenic *Candida* species were found to cleave a wide range of proteins involved in structural and immunological defense, such as collagen, fibronectin and IgG heavy chains[1]. Thus, Saps are assumed to participate in adhesion to host surfaces and invasion into the host tissues[5–8]. The crystallographic structures of two closely related aspartic proteinases from *C. albicans* and *C. tropicalis* have been reported[9–11]. These structures were found to share the archetypic fold and overall topology of classic aspartic proteinases, with several distinguishing features. The substrate specificity studies revealed a pepsin-like character of secreted proteinases from *C. albicans*, *C. tropicalis* and *C. parapsilosis*[12,13].

C. parapsilosis is often isolated from infected skin and nails, and rather occasionally from mucosal infections. It is also reported as a causative agent of bloodstream infections, mostly in patients receiving parenteral nutrition or in connection with persistent central venous catheter[14,15]. Although it is a medically important fungus, studies of the virulence and experimental pathogenicity of *C. parapsilosis* are relatively infrequent. Two genes coding for putative secreted aspartic proteinases have been found in *C. parapsilosis* and designated *ACPR* and *ACPL* (ref.[16]). In the study published by Fusek *et al.*[17], the respective gene products have been denominated CPAP#1 and CPAP#2. Since new genes coding for secreted aspartic proteinases have been found in *C. albicans* and *C. tropicalis*, a new nomenclature has been suggested for this class of enzymes[18]. Thus, the Saps of *C. parapsilosis* will be denominated Sapp1p and Sapp2p, and the relevant genes *SAPP1* and *SAPP2*.

The percentage of identical residues between Sapp1p and Sapp2p is 53%, and 15% of residues are strongly similar. Sapp1p consists of 340 amino acid residues, while Sapp2p of 351 ones. The isoelectric points are 5.3 and 6.4 for Sapp1p and Sapp2p, respectively[17]. With respect to Sapp2p, however,

Abbreviations: BSA, bovine serum albumin; PVDF, polyvinylidene fluoride; SAP, gene encoding secreted aspartic protease; Sap, secreted aspartic protease; Saplp, secreted aspartic protease of *Candida lusitaniae*, Sapp1p, secreted aspartic protease 1 of *Candida parapsilosis*; SE-Sephadex, Sulfoethyl Sephadex; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; YCB, yeast carbon base.

controversial results have been published. De Viragh and colleagues[16] observed only secretion of Sapp1p (*ACPR* gene product) by *C. parapsilosis* in the BSA-supplemented medium. They concluded that *SAPP2P* (= *ACPL*) is either a pseudogene or a gene expressed under not identified conditions. By contrast, Fusek *et al.*[17] found and described a protein of Sapp2p N-terminal sequence obtained in the purification of extracellular aspartic proteinase from the supernatant of culture grown in a liquid medium containing bovine serum albumin.

C. lusitaniae is a species with a low incidence in healthy people. It emerges mainly as a nosocomial pathogen in immunocompromised hosts. Most of the clinical isolates have been recovered from blood, urine, respiratory tract and central venous catether[14,19–21]. *C. lusitaniae* may present therapeutic difficulties, because it is able to develop antifungal resistance *in vivo* during therapy. Acquisition of resistance to amphotericin B is associated with a morphological switch[22]. Nowadays, clinical isolates of *C. lusitaniae* are relatively infrequent in comparison with other *Candida* species. However, the antifungal resistance of *C. lusitaniae* may cause increased occurrence of this species in future. Current studies on *C. lusitaniae* are focused mostly on epidemiology, typing, transmission and antifungal susceptibility. Little is known about individual gene products of *C. lusitaniae*. To our knowledge, the present work is the only characterization of secreted proteinase of *C. lusitaniae* (Saplp), except for an inhibition study published by our group elsewhere[23].

In the present study, we characterize extracellular proteinases of two *Candida* species that have not been studied as extensively as Saps of *C. albicans*: we describe enzymatic properties of proteinases secreted by *C. parapsilosis* and *C. lusitaniae*.

EXPERIMENTAL

Materials

BBL® Skim Milk Powder was from Becton–Dickinson, Cockeysville, U.S.A.; Sabouraud dextrose agar and yeast carbon base (YCB) were from HiMedia Laboratories Ltd., India; bovine serum albumin (BSA) was from Imuna, Šarišské Michalany, Slovakia; PVDF membrane and Durapore membrane filters were from Millipore, U.S.A.; SE-Sephadex C-25 was from Pharmacia. The chromogenic peptide Lys-Pro-Ala-Glu-Phe-Phe(NO_2)-Ala-Leu was synthesized in the Institute of Organic Chemistry and Biochemistry.

Yeast Strains and Cultivation Conditions

Eight clinical isolates including seven strains of *C. parapsilosis* (two recovered from blood culture, two from external ear and one each from central venous catether, skin and nail) and one strain of *C. lusitaniae* recovered from upper respiratory tract were obtained from the patients of University Hospital in Olomouc, Czech Republic. All the strains are deposited in the *Candida* collection of the Institute of Microbiology, Faculty of Medicine, Palacký University, Olomouc. Identification at the species level was done by conventional morphological (evaluation of micromorphology on rice extract agar) and biochemical methods – assimilation and fermentation tests[24]. The results of fermentation tests were as follows: glucose +, sucrose + (week), lactose –, maltose + (week), galactose + (after 3 weeks of incubation at 30 °C). The assimilation tests were performed using the kit ID 32C (bioMerieux, Lyon, France).

Yeast strains were stored in 20% (w/v) BBL® Skim Milk Powder at –70 °C until tested.

The strains were streaked onto Sabouraud dextrose agar. Single colonies from plates were inoculated into 1.2% yeast carbon base. pH of the medium was adjusted to 3 for the cultivation of *C. parapsilosis* and to 4 for that of *C. lusitaniae*. The medium was supplemented with filter-sterilized BSA (final concentration 0.2%). Cultivation was performed at 30 °C in a rotation shaker. The overnight culture was used for inoculation of larger volumes of the same medium (usually 0.5 to 1.5 l). The yeast were cultivated until early stationary phase (OD_{550} > 10) and harvested by centrifugation (10 000 *g*, 15 min, Beckman centrifuge, JA-14 rotor). The supernatant was filtered using Durapore membrane filters. The cell-free supernatants containing secreted proteinases were used for further purification.

Purification of the Secreted Proteinases

The cell-free supernatants were dialyzed against 15 mM sodium citrate buffer (pH 3.5) and applied to the SE-Sephadex C-25 column equilibrated in the same buffer. Proteinases were eluted using salt gradient (0–1 M NaCl in15 mM sodium citrate buffer pH 3.5). The fractions containing active proteinase were collected and analyzed.

Proteolytic Activity Assays

Proteolytic activity in the cell-free supernatants as well as the activity of purified enzyme preparations were determined by cleavage of a chromogenic peptide substrate Lys-Pro-Ala-Glu-Phe*-Phe(NO_2)-Ala-Leu (ref.[25]) (the scissile bond is indicated by asterisk). The reaction was performed at 37 °C in 100 mM sodium acetate buffer (pH 3.7). Typically, substrate was added to 1 ml of the buffer, so that the final concentration was 32 μmol/l. Reaction was started by addition of 10 μl of an enzyme sample. Cleavage was monitored using spectrophotometer (Aminco DW 2000) as a decrease in absorbance at 300 nm during 5 min. Each data point represents an average of three activity values determined with the error reflecting the standard deviation of the used method.

Dependence of the proteolytic activity on pH was examined in a similar manner. Final concentration of enzyme was 1.5 nmol/l for Sapp1p and 6.8 nmol/l for Saplp. Cleavage was examined in 100 mM sodium citrate buffer adjusted to various pH values ranging from 2 to 6. Reaction was started by the addition of substrate (final concentration 40 μmol/l), after 10 min of preincubation of the enzyme in an appropriate buffer.

For the temperature-optimum determination, enzymes of the same concentration as for the pH dependence measurements were added to 100 mM sodium acetate buffer (pH 3.75)

and preincubated at an appropriate temperature for 10 min. The temperature in the cuvette holder was adjusted to the appropriate value as well. Reaction was started by the addition of substrate (final concentration 40 μmol/l). The highest activity was taken as 100%.

SDS-PAGE, N-Terminal Sequencing and Isoelectric Focusing

SDS-PAGE was performed using 10% (w/v) polyacrylamide gels. Where necessary, protein samples for electrophoresis were concentrated from cell-free supernatants by acetone precipitation: samples were mixed with ice-cold acetone (final concentration of acetone was 80%), incubated in freezer for 30–60 min and centrifuged. The sediment was washed with acetone and dried. For the N-terminal sequence analysis, the samples were transferred from gels to PVDF membrane using a Hoefer semi-dry blotting unit. The membranes were stained with Coommassie Brilliant Blue, individual bands were cut and sequenced in ABI Procise protein sequencer 491. Isoelectric focusing was performed using a Mini-PROTEAN II Tube Cell system according to the instructions of manufacturer. pH gradient in the range between 10 and 3 was used.

RESULTS AND DISCUSSION

Yeast Cultivation and Proteinase Purification

In order to induce proteinase production, bovine serum albumine (BSA) was present as a sole nitrogen source during cultivation of both *C. parapsilosis* and *C. lusitaniae*. Under the optimum cultivation conditions, BSA was almost completely degraded and utilized at the end of logarithmic phase. Thus, in the stationary phase, the secreted proteinases became major protein fraction present in the medium. The optimum cultivation temperatures were 30–37 °C, and optimum pH of the medium was 3 for *C. parapsilosis* and 4 for *C. lusitaniae*. At suboptimum conditions (temperatures below 29 °C, pH above 5), the lag phase was prolonged, BSA degradation not completed and the proteinase yield was significantly decreased. These observations correspond to the results reported by others for cultivation of *C. albicans*[26].

The ion-exchange chromatography of cell-free culture supernatants was performed in order to remove the remaining of BSA fragments (Fig. 1). Fractions with the highest proteolytic activity were pooled. For both the proteinases studied here, one chromatographic step was sufficient for achieving an electrophoretically homogenous active enzyme preparation (Fig. 2). The SDS-PAGE analysis of active Saplp fractions revealed a protein of a molecular weight approximately 42 000. It is similar to molecular weight of Sap2p, a major secreted proteinase of *C. albicans*.

In articles published by other groups, chromatography on DEAE-Sephadex at pH 5.6 is often mentioned as a tool for successful purification of the

Sap2p isoenzyme of *C. albicans* and extracellular proteinase of *C. tropicalis*[12,25,27,28]. We have tested this approach for purification of Sap1p and Sapp1p, but the results were unsatisfactory.

N-Terminal Sequence Analysis

To further confirm homogeneity of the Sap preparations, the purified active samples were subjected to N-terminal sequencing. In the samples of

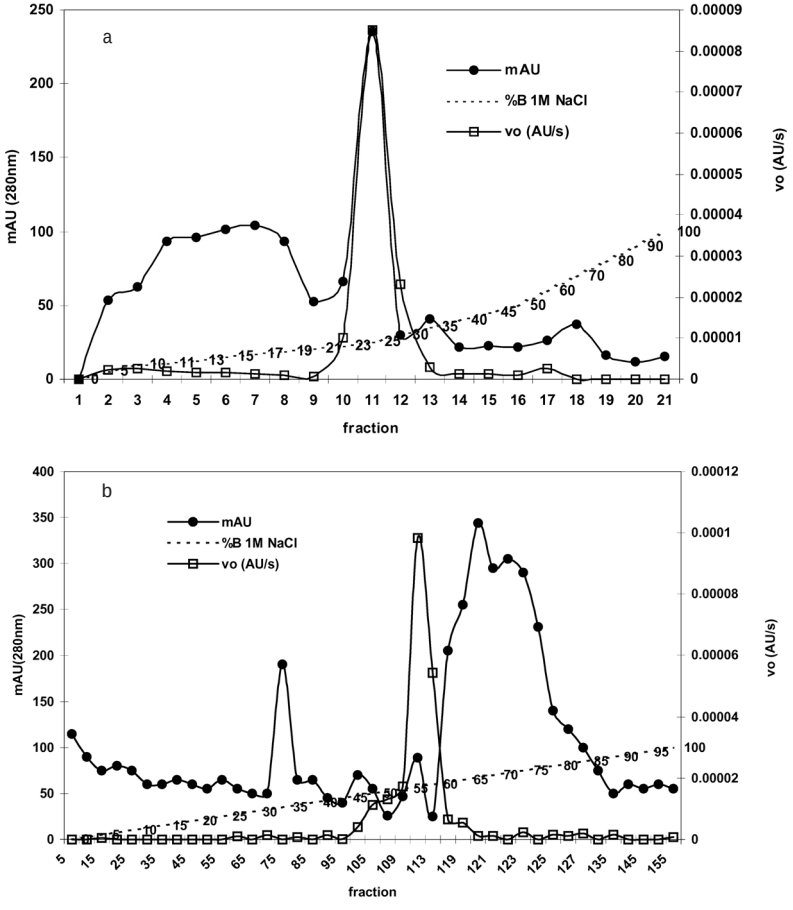

FIG. 1

Cation-exchange chromatography of the cell-free supernatants from the cultivation of *C. parapsilosis* (a) and *C. lusitaniae* (b). The Saps were eluted using a 0–1 M NaCl gradient. Proteolytic activity, detected using the peptide substrate Lys-Pro-Ala-Glu-Phe*-Phe(NO_2)-Ala-Leu, was expressed as initial velocity (v_0) determined as a decrease of absorbance (AU) at 300 nm/s

proteinase from *C. lusitaniae*, the analysis revealed a stretch of 25 amino acids: GSYPETLKDVDDVSYVVDIYKGSDK- (ref.[23]).

The N-terminal sequence of the purified proteinase secreted by *C. parapsilosis* was DSISL-, which corresponds to Sapp1p (ref.[16]). In order to examine whether Sapp2p was also secreted, N-terminal sequencing was carried out using cell-free culture supernatants. All of the seven strains studied yielded only the Sapp1p N-terminal sequence. The comparison of the N-terminal 25 residues of Saps of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae* was recently published by Pichová *et al.*[23]. Saplp and Saplp have two identical residues (Ser 2 and Leu 7) and 7 similar amino acid residues within this sequence.

The isoenzymes of secreted proteinases of *C. parapsilosis* were reported to differ in isoelectric points. p*I* 5.3 and 6.4 were experimentally determined for Sapp1p and Sapp2p, respectively[17]. Therefore culture supernatants obtained from the cultivation of *C. parapsilosis* were analyzed using also isoelectric focusing. Our analyses revealed only one spot (data not shown). Thus, the results obtained in this study support rather the assumption of de Viragh *et al.*[16], that Sapp2p may be expressed under as yet unidentified conditions or *SAPP2P* may correspond to an untranslated genomic region. The amount of Sapp2p detected by Fusek *et al.*[17] was approximately four times lower than that of Sapp1p. Thus, it is also possible that in our experiments, Sapp2p was secreted, but its concentration in the medium was too low for the detection and analysis. The difference in the expression of Sapps could be also caused by minor variations in the growth conditions or/and by intraspecies variability of *C. parapsilosis*.

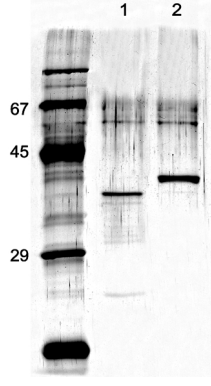

FIG. 2
SDS-PAGE of the isolated secreted aspartic proteinases of *C. parapsilosis* (Sapplp, lane 1) and *C. lusitaniae* (Saplp, lane 2). Molecular size markers (in kDa) are indicated on the left side

Effect of pH on the Proteolytic Activity

Both Sapp1p and Saplp cleave readily the substrate designed originally for pepsin Lys-Pro-Ala-Glu-Phe*-Phe(NO_2)-Ala-Leu, at the site indicated by asterisk. Both the proteinases are inhibited by pepstatin[23]. These results indicate that Saplp is an aspartic proteinase, as expected. Cleavage of the peptide substrate by Sapp1p and Saplp was examined using buffers of different pH values. The results are illustrated in Fig. 3. Maximum activity of Sapp1p was observed at pH ranging approximately from 3.2 to 4. At pH above 4.5, the activity of Sapp1p dropped significantly. Saplp displayed highest activity at pH 3.5–3.75, while at pH below 2.5 and above 4.5 the enzyme lost its activity. These results were not surprising, since low pH optimum is one of the general features of aspartic proteinases. Furthermore, Fusek *et al.*[17] reported the most efficient cleavage of BSA by Sapp1p at pH 3–3.5. The optimum pH value for the activity of individual proteinases corresponds to the optimum pH for the cultivation of the relevant *Candida* species on the proteinaceous medium, where the culture growth depends on the ability to utilize external protein as a source of nitrogen.

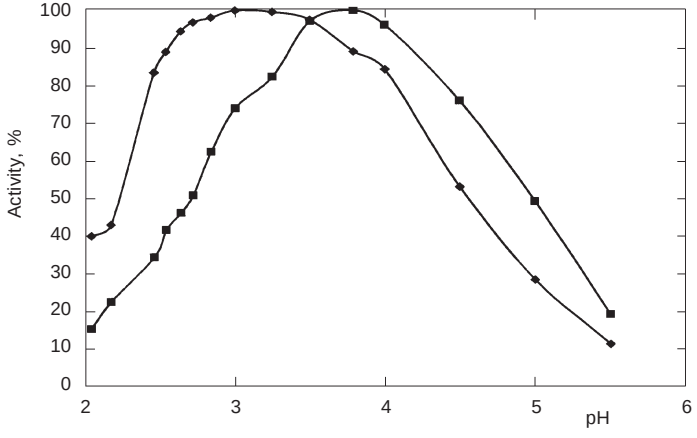

FIG. 3

Effect of pH on the activity of secreted aspartic proteinase of *C. parapsilosis* (Sapp1p, ◆) and *C. lusitaniae* (Saplp, ■). The reactions were performed at 37 °C, in 1 ml of 100 mM sodium citrate buffer of appropriate pH. Final concentration of enzymes was 1.5 nmol/l (Sapp1p) and 6.8 nmol/l (Saplp). The reactions were started by the addition of substrate Lys-Pro-Ala-Glu-Phe*-Phe(NO_2)-Ala-Leu (final concentration 40 μmol/l) after 10 min of preincubation of the enzyme in the appropriate buffer. The cleavage was monitored at 300 nm with a spectrophotometer Aminco

Effect of Temperature on the Proteolytic Activity

Proteolytic activity (at pH 3.75) of the individual proteinases was examined at temperatures ranging from 20 to 60 °C. As illustrated in Fig. 4, substantial differences between both enzymes were observed. Saplp showed a plateau of maximum activity between 27–40 °C. At temperatures above 45 °C, the Saplp activity decreased sharply. These results resemble the data published by Smolenski *et al.*[28] on the properties of Saps from *C. albicans*. In contrast, maximum activity of Sapp1p was detected at 45 °C. Sapp1p cleaves the substrate with 70% efficiency even at 55 °C, while Saplp is inactivated at this temperature.

Stability of both enzymes was monitored at 37 °C and pH 3.5 (Fig. 5). After 60 h of incubation under these conditions, both enzymes retained approximately 85% of their activity. Both proteinases lost 50% of activity after 8 days. Thus, these enzymes proved to be stable at optimum pH for their activity and at physiological temperature. Thermal stability of Sapp1p was examined also at 50 °C. Figure 6 shows that the enzyme activity decreased to 50% after 5–6 h.

Optimum temperature of Sapp1p was relatively high, particularly with respect to the fact, that *C. parapsilosis* is a human pathogen. It is difficult to

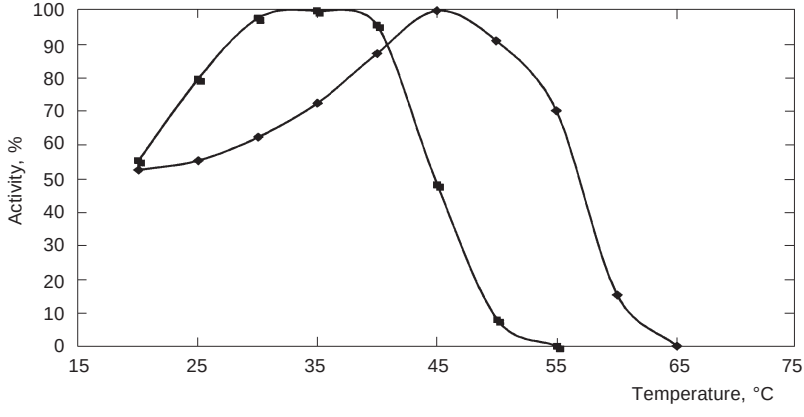

FIG. 4
Effect of temperature on the activity of secreted aspartic proteinase. The enzymes were added to 1 ml of 100 mM sodium acetate buffer (pH 3.75) and incubated for 10 min at an appropriate temperature. Final concentration of enzymes was 1.5 nmol/l (Sapp1p, ◆) and 6.8 nmol/l (Saplp, ■). The reaction was started by the addition of substrate Lys-Pro-Ala-Glu-Phe*-Phe(NO_2)Ala-Leu (final concentration 40 μmol/l). The cleavage of substrate was monitored spectrophotometrically at 300 nm

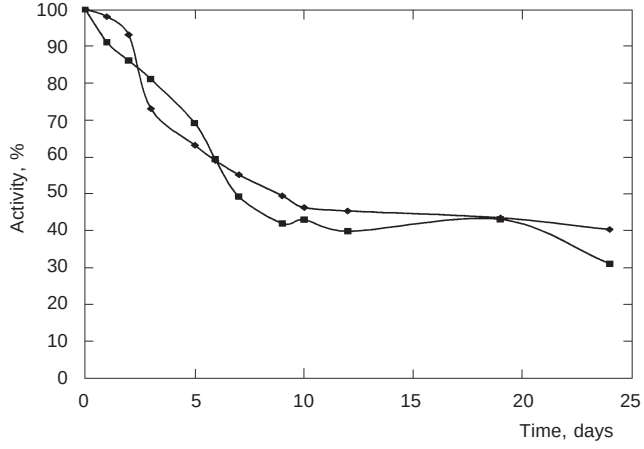

FIG. 5

Stability of secreted aspartic proteinase of *C. parapsilosis* (Sapp1p, ◆) and *C. lusitaniae* (Saplp, ■) at 37 °C. The 1.5 nM and 6.8 nM solution of Sapp1p and Saplp, respectively, in 100 mM sodium citrate buffer (pH 3.5) was incubated at 37 °C. For the activity measurements, 1-ml aliquots were transferred to the cuvette. The reaction was started by the addition of substrate at final concentration 32 μmol/l and monitored spectrophotometrically at 300 nm

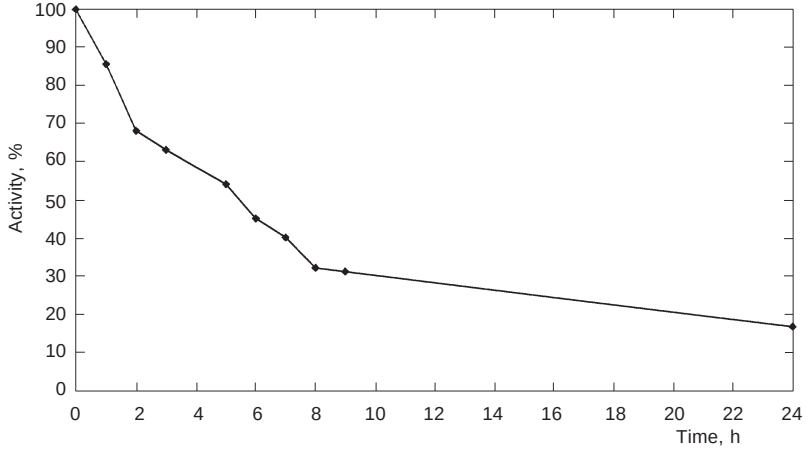

FIG. 6

Stability of secreted aspartic proteinase of *C. parapsilosis* (Sapp1p) at 50 °C. Sapp1p (final concentration 1.5 nmol/l) was incubated in 100 mM sodium acetate buffer (pH 3.25) at 50 °C. At appropriate times, 1-ml aliquots were transferred to the cuvette and the reaction was started by the addition of substrate at final concentration 32 μmol/l. The cleavage was monitored spectrophotometrically at 300 nm

speculate about a possible role of structural features in the Sapp1p activity, since the crystal structure of Sapp1p is not known. The high temperature required for maximum enzyme activity or high thermal stability is not uncommon among acid proteinases, particularly from microbial sources. It was observed *e.g.* in aspartic proteinase from *Rhizomucor miehei*[29] or *Phycomyces blakesleeanus*[30]. In these cases, however, the high optimum temperature and thermal stability has been attributed to glycosylation of the molecule, which has not been found in Sapp1p. Thermopsin, acid proteinase from archaebacterium *Sulfolobus acidocaldarius* displays maximum activity at 90 °C, but its sequence is distinct from other members of the aspartic proteinase family[31]. Thus, solution of Sapp1p structure and its comparison with the structures of Saps from *C. albicans* will facilitate the understanding of the above-mentioned enzymatic properties.

CONCLUSIONS

Our study involving seven clinical isolates of *C. parapsilosis* and one isolate of *C. lusitaniae* has confirmed that both species secrete only one proteolytically active aspartic proteinase during their cultivation in liquid medium containing BSA as the source of nitrogen. Both proteinases show the maximum proteolytic activity at low pH which is typical for other aspartic proteinases of other *Candida* species[13]. The stability of Saplp and Sapp1p under these conditions (low pH, 37 °C) is comparable with those of Saps secreted by *C. albicans*[28]. Interestingly, proteinase secreted by *C. parapsilosis* displays unusually high optimum temperature. Since *C. parapsilosis* is a human pathogen, optimum temperatures for its enzymes are expected to be close to 37 °C. Thus, it would be interesting to investigate which structural motifs are responsible for the high optimum temperature and thermal stability of Sapp1p.

Biochemical characterization of secreted proteinases of *C. parapsilosis* and *C. lusitaniae* provides a basis for the diagnosis and typing of *Candida* species that develop a higher resistance to polyenes and azoles, used for a treatment of candidiosis. The detailed characterization contributes also to the design of tight binding inhibitors of Saps that represent a new specific target for anticandidal agents.

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