Cloning and characterization of Sapp2p, the second aspartic proteinase isoenzyme from Candida parapsilosis

Michaela Merkerová, Jiří Dostál, Martin Hradilek, Iva Pichová & Olga Hrušková-Heidingsfeldová

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Correspondence: Olga Hrušková-Heidingsfeldová, Institute of Organic Chemistry and Biochemistry, Flemingovo nám. 2, Prague 6, 166 10, Czech Republic. Tel.: +420 220 183 249; fax: +420 224 310 090; e-mail: olga-hh@uochb.cas.cz

Present address: Michaela Merkerová, Institute of Hematology and Blood Transfusion, U Nemocnice 1, Prague 2, 128 20, Czech Republic.

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Abstract
The human fungal pathogen Candida parapsilosis possesses at least three genes encoding secreted aspartic proteinases. Whereas the Sapp1p isoenzyme has already been biochemically characterized, the SAPP2 and SAPP3 gene products have not. The Sapp2p precursor, pro-Sapp2p, was therefore expressed in Escherichia coli and purified. Autoactivation of pro-Sapp2p in acidic conditions was inefficient and resulted in a protein extended by eight amino acids at the N-terminus (Sapp2p^8). The correct promature junction KR/SSPSS was cleaved by trypsin or by a membrane-bound Kex2-like proteinase from Candida parapsilosis. The mature Sapp2p obtained by the assisted activation was proteolytically active. Its activity was more than twofold higher than that of the self-processed protein species Sapp2p^8, as measured by the hemoglobin cleavage test. The substrate specificity of Sapp2p differs from that of Sapp1p. Peptides containing aromatic residues in the P1 and P1’ positions are cleaved poorly by Sapp2p. A fluorogenic substrate was synthesized to facilitate further studies.

Introduction
Candida parapsilosis is an opportunistic fungal pathogen, associated mainly with bloodstream and skin infections. It also represents a serious threat for critically ill neonates. According to recent surveys, C. parapsilosis is the second or third most commonly isolated Candida species from blood in Europe, Canada, and Latin America (Krcmery & Kovacicova, 2000; Pfaller et al., 2001; Swinne et al., 2004; Peman et al., 2005). Moreover, C. parapsilosis is a main cause of nosocomial hospital infection by yeasts (Bonassoli et al., 2005). Despite these facts, virulence determinants of C. parapsilosis have not been sufficiently characterized. It is routinely assumed that virulence determinants of all pathogenic Candida species are similar to those of Candida albicans. As C. albicans is the most frequent human fungal pathogen, it has attracted more attention than any other pathogenic yeast species, and its virulence determinants, including yeast–hypha morphogenesis, secretion of hydrolytic enzymes, and specific metabolic traits, are being extensively studied. It is likely that many non-albicans species share some common virulence determinants with C. albicans, but also have a distinct repertoire of specific virulence traits (Haynes, 2001).

Secretion of aspartic proteinases (Saps) is one of the well-known virulence factors of C. albicans. Most of the clinical isolates of C. parapsilosis, Candida tropicalis, Candida dubliniensis and Candida lusitaniae are proteinase producers as well (Monod et al., 1993; Dostál et al., 2003; Naglik et al., 2003) and usually have more than one Sap isoenzyme (De Virag et al., 1993; Monod et al., 1994; Zaugg et al., 2001). However, the role of Saps in the virulence of non-albicans Candida has not been fully clarified. Each of the Sap-producing Candida species has one Sap isoenzyme, which is a major product secreted in vitro, elicited in nitrogen-limited media by the presence of an exogenous protein as the sole nitrogen source. These enzymes, Sap2p from C. albicans, Sapt1p from C. tropicalis, and Sapp1p from C. parapsilosis, have been purified from the yeast culture supernatants, biochemically characterized, and crystallized (Fusek et al., 1994; Cutfield et al., 1995; Abad-Zapatero et al., 1996; Symersky et al., 1997; Pichová et al., 2001; Naglik et al., 2003). Other Sap isoenzymes, however, have received much
less attention, and those of non-*albicans* *Candida* in particular remain almost unknown, the only available information being their gene sequences in databases.

Two genes coding for Saps have been found in *C. parapsilosis* and designated *ACPR* and *ACP* (De Viragh et al., 1993), and the respective gene products were designated CPAP#1 and CPAP#2 (Fusek et al., 1993). According to the new nomenclature, these genes were renamed SAPP1 and SAPP2, and the respective proteins were renamed Sapp1p and Sapp2p. The third SAPP gene (SAPP3) has been identified recently by Zaugg and Monod; its accession number in the NCBI database is AF339513. The Sapp1p isoenzyme has been isolated from *C. parapsilosis* culture supernatants or prepared by expression in *Escherichia coli* and characterized on an enzymologic level (Fusek et al., 1994; Hrušková-Heidingsfeldová et al., 2001; Pichová et al., 2001; Dostál et al., 2005). By contrast, the SAPP2 gene product remains rather enigmatic. The putative Sapp2p isoenzyme was not detected in culture supernatants, and the SAPP2 gene was not able to complement a *C. tropicalis* mutant defective in secreted aspartic proteinase (De Viragh et al., 1993). Therefore, it has been hypothesized that SAPP2 could be a pseudogene, or that it might be expressed under as yet unidentified conditions and serve an unknown function. In contrast to these observations, Fusek et al. (1993) function reported isolation of Sapp2p from a *C. parapsilosis* culture medium containing exogenous protein as the sole nitrogen source. Although Sapp2p constituted only about 20% of total enzyme recovered from the medium, it was shown to be expressed and secreted. However, these results have not been reproduced by others, and the characteristics of the potential SAPP2 gene product remained questionable (Hrušková-Heidingsfeldová et al., 2001).

The aims of the present study were to elucidate whether the SAPP2 gene product displays proteolytic activity and to examine the properties of the putative Sapp2p isoenzyme.

**Materials and methods**

**Strains and growth conditions**

*Candida parapsilosis* strains CP 386/IDE98 and CP 924/IDE03 were isolated from ear and urine of patients in the hospital of the Faculty of Medicine, Palacky University, Olomouc, Czech Republic. Identification of the isolates was performed by standard procedures, including biochemical assays with commercial kits [the Auxacolor system (Bio-Rad) and the ID 32C system (bioMérieux)]. The final assignment of the isolates to the *C. parapsilosis* group was done by PCR amplification of the fragments of three secreted proteinase genes and a fragment of the secondary alcohol dehydrogenase, followed by restriction analysis, as described by Tavanti et al. (2005).

The yeasts were cultivated in YPD (1% yeast extract, 2% peptone, 2% glucose) or YCB-BSA [1.2% yeast carbon base, 0.2% bovine serum albumin (BSA), pH 3.5] at 30 °C. *Escherichia coli* strains DH5α and BL21(IDE3) were used for DNA manipulation and protein expression, respectively.

**Cloning and expression of pro-Sapp2p**

DNA from *C. parapsilosis* was isolated as described elsewhere (Hoffman & Winston, 1987). The Sapp2p precursor was amplified using the primers 5′-GACCCCGGTACCATGGATGATAAC-3′ and 5′-TAACTCGAGTTATATTGAATATAACGATTGTA-3′, which created an NcoI site upstream of the coding sequence and an Xhol site downstream of the native stop codon.

The 1179-bp PCR product was cut with NcoI and Xhol and inserted into the polylinker of pET-24d(+) plasmid (Novagen), yielding the expression vector pET-24d(+)-PRO-SAPP2. Fidelity of the insertion was verified by DNA sequencing.

*Escherichia coli* BL21(IDE3) transformed with pET-24d(+)-PRO-SAPP2 was cultivated in kanamycin-supplemented Luria-Bertani medium at 37 °C to OD_{550 nm} = 0.8. Then, production of pro-Sapp2p was initiated by addition of isopropyl-1-thio-D-galactopyranoside (IPTG) to a final concentration of 1 mM. The cells were cultivated for another 3 h after induction, and harvested by centrifugation at 4600 g for 10 min.

**pro-Sapp2p purification**

Isolation and purification of pro-Sapp2p was performed as described (Lin et al., 1993; Dostál et al., 2005). Briefly, the cells were resuspended in TN buffer (100 mM Tris-Cl, pH 7.4, 150 mM NaCl) and disintegrated by addition of lysozyme (1 mg·ml⁻¹ of wet biomass), freezing and thawing, sonication and addition of deoxycholate to a final concentration of 0.1%. Inclusion bodies containing pro-Sapp2p were separated by centrifugation of the disintegrated cells at 9500 g for 15 min and washed three times with wash buffer (100 mM Tris-Cl, pH 7.0, 2 M urea, 5 mM EDTA, 2% Triton). Inclusion bodies were solubilized in 50 mM Tris-Cl buffer, pH 8.0, containing 1 mM glycine, 1 mM EDTA, 8 M urea and 100 mM mercaptoethanol.

Purification of pro-Sapp2p from the solubilized inclusion bodies was carried out using fast protein liquid chromatography (FPLC) on a MonoS column equilibrated in 20 mM sodium acetate buffer, pH 3.5, containing 6 M urea. Proteins were eluted with an NaCl gradient. Samples containing pro-Sapp2p were dialyzed into 20 mM Tris-Cl buffer, pH 7.2, and purified using a MonoQ column equilibrated in the same buffer. Elution of pro-Sapp2p was also performed using an NaCl gradient.
Autoactivation of pro-Sapp2p

Autoactivation of pro-Sapp2p was performed similarly to autoactivation of pro-Sapp1p (Dostál et al., 2005). Purified pro-Sapp2p (0.3–0.4 mg mL⁻¹) in 20 mM Tris-Cl, pH 7.2, was acidified by direct, stepwise addition of 0.5 M HCl. The mixture was stirred and the pH was monitored. When the pH reached the desired values, the temperature was adjusted to 37 °C. Aliquots were taken at different time points, and the precursor processing was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The propeptide released by the self-processing was removed using gel chromatography on a Hi load™ 16/60 Superdex™ column (Amersham) equilibrated in 100 mM sodium acetate buffer, pH 3.5.

Processing of pro-Sapp2p by trypsin

Purified pro-Sapp2p (0.2 mg mL⁻¹) in 20 mM Tris-Cl, pH 7.2 was incubated with bovine trypsin (Fluka). The final trypsin concentration was 0.5 μg mL⁻¹. The reaction proceeded for 1 h at room temperature and was stopped by addition of phenylmethylsulfonyl fluoride (PMSF). The propeptide released by trypsin cleavage was removed using gel chromatography, as described above.

Processing of pro-Sapp2p by Sapp1p

Purified Sapp1p was prepared from the C. parapsilosis cell-free culture supernatant as described (Hrusková-Heidingsfeldová et al., 2001). Pro-Sapp2p (0.3–0.5 mg mL⁻¹) was dialyzed to 100 mM sodium acetate buffer, pH 3.5. Then, Sapp1p was added to a final concentration of 0.5 μg mL⁻¹. The mixture was incubated at 37 °C for 20 h. Aliquots for SDS-PAGE analysis were taken at different time points.

Preparation of the membrane fraction from C. parapsilosis

The membrane fraction was prepared as described (Achstetter & Wolf, 1985; Dostál et al., 2005). Candida parapsilosis cells were washed with water and with 100 mM Tris-Cl, pH 7.2, and disintegrated using a French press. The cell extract was centrifuged for 10 min at 3000 g. The resulting supernatant was centrifuged for 35 min at 150 000 g. The pellet was resuspended in three times the starting volume of 20 mM Tris-Cl, pH 7.5, containing 200 mM KCl. The membranes were sedimented at 150 000 g for 40 min. This procedure was repeated using six times the starting volume of the 20 mM Tris-Cl buffer, pH 7.5, containing 200 mM KCl, and sedimentation of the membranes was carried out at 260 000 g. The membranes were resuspended in 20 mM Tris-Cl, pH 7.5, and stored at −80 °C. The protein concentration in these preparations was 0.5–0.7 mg mL⁻¹, as detected by the Bradford assay (Bradford, 1976).

Processing of pro-Sapp2p by a membrane-bound protease

The cleavage of pro-Sapp2p by a membrane-bound protease was performed as described by Dostál et al. (2005). Twenty microliters of pro-Sapp2p solution (0.5 mg mL⁻¹) was mixed with 10 μL suspension of the membrane fraction and 130 μL of 200 mM Bis-Tris buffer, pH 6.5–7, containing 1 mM CaCl₂, 0.1% Triton X-100, 4 mM bestatin, and 2 μM pepstatin A. The reaction mixture was incubated at 37 °C overnight, and the reaction was stopped by adding electrophoresis loading buffer and incubating in boiling water for 2 min. The cleavage was then analyzed using SDS-PAGE and Western blotting. Primary antibodies were anti-Sapp1p, which crossreact with Sapp2p (there is 53.3% identity and 79.1% similarity between the two protease isoenzymes). The antibodies were produced in rabbits at the Institute of Physiology, the Academy of Sciences of the Czech Republic, Prague. Secondary antibodies were peroxidase-labeled swine antirabbit immunoglobulins (Sevapharma, Prague, Czech Republic). Sapp2p bands were visualized by incubation with BM blue POD substrate from Roche (Manheim, Germany).

N-terminal sequencing

Proteins were separated by SDS-PAGE and transferred to poly (vinylidene difluoride) membranes using electroblotting apparatus. After Coomassie blue staining, appropriate bands were cut and their N-terminal sequences were determined by Edman degradation using an Applied Biosystems Procise sequencer.

Assays of proteolytic activity

Cleavage of peptides was performed in 100 mM sodium acetate, 100 mM sodium citrate or McIlvaine buffer adjusted to a required pH ranging from 2 to 6. Typically, 10 μL of a peptide stock solution (3–5 mM) was mixed with 100 μL of an appropriate buffer and 30 μL of Sapp2p sample (0.04 mg mL⁻¹). After an overnight incubation at 37 °C, the reaction was stopped by addition of 20 μL of 20% trifluoroacetic acid (TFA). Reaction products were analyzed using HPLC on a Nova-Pak C-18 column. A linear methanol gradient was used for elution, and the cleavage products were detected spectrophotometrically at 220 nm.

Cleavage of BSA was performed in 100 mM sodium acetate buffer, pH 3, or McIlvaine buffer, pH 2–6. The final concentration of BSA was 0.2 mg mL⁻¹, and the final concentration of purified Sapp2p sample was c. 35 μg mL⁻¹ in a
The concentrations of Sapp2p, Sapp2p-m, and Sapp1p were typically contained 5 mg mL\(^{-1}\), 0.04 mg mL\(^{-1}\), and 250 \(\mu\)g mg\(^{-1}\) hemoglobin solution in water. The concentrations of Sapp2p, Sapp2p\(^{+8}\) and Sapp1p were adjusted to the same value in each experiment. The typical proteinase concentration in the samples tested was 0.04 mg mL\(^{-1}\). The mixture was incubated at 37 °C for 8 h, and the reaction was stopped by addition of 200 \(\mu\)L of 10% trichloroacetic acid and centrifugation (15 min, 13 000 g). The enzyme activity is proportional to the absorbance of the supernatant measured at 280 nm. The Anson test was also used to examine the effect of pH on Sapp2p activity. For these experiments, the sodium citrate buffer was replaced with Mcllvaine buffer, with a pH ranging from 2 to 6, and the incubation time was 60 min. In order to examine the effect of temperature on Sapp2p activity, the Anson test was performed using 100 mM sodium citrate buffer, pH 4. The reaction mixture was divided into aliquots that were incubated at temperatures ranging from 4 to 55 °C.

The Sapp2p activity assay using the fluorogenic peptide Dabcyl-Glu-His-Val-Leu-Val-Glu-EDANS was performed in 100 mM sodium acetate buffer, pH 3.75, at 37 °C. The reaction mixture of a total volume of 150 \(\mu\)L typically contained 5 \(\mu\)L of the substrate stock solution (5 mg mL\(^{-1}\) in dimethyl sulfoxide) and 20 \(\mu\)L\(^{-1}\) of the enzyme solution (0.05 mg mL\(^{-1}\)). The cleavage was monitored either using a plate reader or using HPLC (Nova-Pak C18 column) equipped with a fluorescence detector. The excitation wavelength was 360 nm, and the emission wavelength was 485 nm. The reactions proceeded for up to 10 min in the plate reader assays. For the HPLC analysis, the incubation times ranged from 2 to 16 h, depending on the quality of the enzyme preparation. The reaction was stopped by addition of 20 \(\mu\)L of 20% TFA.

The Anson test (Anson, 1938; Fusek et al., 1993), i.e. digestion of hemoglobin by proteinase samples, was performed as follows. Ten microliters of a proteinase sample was mixed with 750 \(\mu\)L of 100 mM sodium citrate buffer, pH 3.4, and 250 \(\mu\)L of 4% (w/v) hemoglobin solution in water. The reaction mixture was divided into aliquots that were incubated at temperatures ranging from 4 to 55 °C for 8 h, and the reaction was stopped by addition of 200 \(\mu\)L of 10% trichloroacetic acid and centrifugation (15 min, 13 000 g). The enzyme activity is proportional to the absorbance of the supernatant measured at 280 nm. The Anson test was also used to examine the effect of pH on Sapp2p activity. For these experiments, the sodium citrate buffer was replaced with Mcllvaine buffer, with a pH ranging from 2 to 6, and the incubation time was 60 min. In order to examine the effect of temperature on Sapp2p activity, the Anson test was performed using 100 mM sodium citrate buffer, pH 4. The reaction mixture was divided into aliquots that were incubated at temperatures ranging from 4 to 55 °C.

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**Synthesis of fluorogenic substrates for Sapp2p activity testing**

Peptides Dabcyl-Pro-Lys(Boc)-Val-Glu(OtBu)-Leu-Thr(tBu)-Gly-Glu(OtBu)-OH and Dabcyl-Glu(OtBu)-His(Trt)-Val-Lys(Boc)-Leu-Val-Glu(OtBu)-OH were prepared via a 9-fluorenylmethoxycarbonyl (Fmoc)/tert-butyl (tBu) strategy using 2-chlorotriyl chloride resin (Fluka; substitution 1.9 mmol Cl g\(^{-1}\) resin). First, Fmoc-Glu(OtBu)-OH (one equivalent) and four equivalents of disopropylethylamine (DIEA) were dissolved in dry dichloromethane (DCM) and attached to the resin. The couplings were carried out with three equivalents of protected amino acid and N,N\(^{\prime}\)-disopropylcarbodiimide/1-hydroxybenzotriazole in dimethylformamide (DMF). Fmoc protecting groups were removed using 20% piperidine in DMF. Fully side-chain-protected peptides were cleaved with a mixture of acetic acid, 2,2,2-trifluoroethanol and DCM (1 : 1 : 3). Crude hydrophilized peptides were dissolved in DMF and added to a solution of 5-(2-aminoethylamino)-1-naphthalenesulfonic acid (1.1 eq.), [(benzotriazol-1-yl)oxy]tris(dimethylamino)phosphonium hexafluorophosphate (1.1 eq.) and DIEA (2.2 eq.). After 12 h at room temperature, mixtures were evaporated. Residues were treated with TFA in the presence of 5% thioanisole, 3% ethanedithiole and 2% anisole as scavengers to remove side-chain protection, and purified by preparative reverse-phase HPLC using a Vydac C18 column.

**Results**

**Expression and purification of Sapp2p precursor**

The Sapp2p precursor was expressed in E. coli in order to address the question of whether the SAPP2 gene product can act as a proteolytic enzyme. Expression of the putative proteinase in a precursor form is preferable, because propeptides often play an important role in the folding of mature domains; aspartic proteinases expressed in bacteria without propeptides may not attain correct structure and activity (Eder & Fersht, 1995; Fusek & Větvíčka, 1995). However, the junction between presequence and prosequence has not been determined in any secreted aspartic proteinase of Candida spp., and thus the precise lengths of the Sap propeptides are not known. A prosegment consisting of 29 residues was sufficient to ensure correct folding of Sapp1p (Dostál et al., 2005). Therefore, the expression vector for pro-Sapp2p was designed similarly. The sequence of the putative Sapp2p mature domain was preceded by 29 amino acids at the N-terminus. The Sapp2p precursor was accumulated in inclusion bodies during the bacterial expression, and the maximum yield of the recombinant protein was obtained 3 h after induction with IPTG. Two chromatographic steps using ion exchange columns were sufficient to obtain a homogeneous protein preparation. The typical yield was 30 mg of purified pro-Sapp2p per liter of bacterial culture.

**Autocatalytic processing of pro-Sapp2p is inefficient**

In order to find an optimal pH for potential autocatalytic processing, the samples of pro-Sapp2p were acidified to pH 2.6, 3, 3.5, 4, 4.5, 5, and 5.5. The cleavage proceeded most efficiently at pH 3.5–4, whereas at pH 5.5 the precursor remained unprocessed. By contrast, complete degradation of pro-Sapp2p occurred at pH values below 3 (Fig. 1). N-terminal analysis of the self-processed protein revealed the sequence ATSEL, alanine being the 39th amino acid.
upstream of the first active aspartate (Fig. 2). However, according to the observations published by others, the N-terminus of Sapp2p is located 31 amino acids upstream of the first catalytic triad Asp-Thr-Gly, and the N-terminal sequence is SSPSS (Fusek et al., 1993). Accordingly, incubation of recombinant pro-Sapp2p in acidic conditions resulted in cleavage of eight amino acids upstream of the putative first amino acid of the mature protein. The resulting protein species was named Sapp2p^+. The incorrect cleavage site, and especially the length of incubation time needed for pro-Sapp2p processing, suggest that self-processing at acidic pH is not the key mechanism of Sapp2p activation.

**pro-Sapp2p can be processed by serine-type proteinases**

The expected promature junction of pro-Sapp2p contains an Arg-Lys motif, which could be a substrate of serine-processing proteinases. The possibility of assisted processing of pro-Sapp2p was therefore tested using bovine trypsin. The precursor was completely processed by trypsin after 1 h of incubation at room temperature, and N-terminal analysis of the cleavage product revealed the expected sequence SSPSS (Fig. 3).

A similar experiment was repeated with a membrane fraction prepared from _C. parapsilosis_ cells, because membrane-bound Kex2-like proteinases are considered as the enzymes responsible for activation of some of the Sap...
Isoenzymes, both in *C. parapsilosis* and in *C. albicans* (Newport & Agabian, 1997; Newport et al., 2003; Dostál et al., 2005). Incubation of pro-Sapp2p with the *C. parapsilosis* membrane fraction also resulted in the protein with the N-terminal sequence SSPSS (Fig. 4.). In this case, however, the precursor was not processed completely, probably due to low stability of the membrane-bound proteinase. The cleavage was inhibited by 1 mM EDTA and 1 mM ZnCl₂, but was not sensitive to PMSF. The same cleavage and inhibition pattern was observed during the study of the activation of Sapp1p zymogen (Dostál et al., 2005). This indicates that both pro-Sapp1p and pro-Sapp2p are processed by the same type of membrane-bound, Kex2-like proteinase.

**Involvement of Sapp1p in pro-Sapp2p processing is unlikely**

Synthetic peptides derived from the processing sites on pro-Sapp2p were prepared and incubated with recombinant Sapp2p activated by trypsin. The peptide PLDVNATSEL, corresponding to the autoprocessing site adjacent to the Sapp2p₁⁸ N-terminus, was cleaved by Sapp2p between asparagine and alanine. The peptide ELSKRSSPS, mapping the correct premature junction, was not hydrolyzed by Sapp2p. These results are in agreement with the mode of pro-Sapp2p self-processing. Nevertheless, the latter peptide was cleaved by the purified authentic Sapp1p, suggesting that this isoenzyme may play a role in the activation of the Sapp2p zymogen.

Pro-Sapp2p was therefore incubated with purified Sapp1p. However, no effect was observed, with the exception of several minor unspecific degradation products (data not shown). These results indicate that pro-Sapp2p processing depends mainly on a membrane-bound proteinase.

**Proteolytic activity of recombinant Sapp2p**

Both Sapp2p⁺⁸ and Sapp2p activated by trypsin were subjected to gel chromatography prior to the enzyme activity testing, in order to remove propeptides that might affect the proteinase activity. Both proteinase species failed to cleave a typical substrate of pepsin-like proteinases Lys-Pro-Ala-Glu-Phe-Phe(p-NO₂)-Ala-Leu. Less than 15% of the peptide present in the reaction mixture was cleaved after an overnight incubation. Moreover, the cleavage occurred at two sites. Similar results were obtained with another two chromogenic peptides: Lys-Ala-Arg-Val-Nle-Phe(p-NO₂)-Glu-Ala-Nle, and Ala-Thr-His-Gln-Val-Tyr- Phe(p-NO₂)-Val-Arg-Lys-Ala.

Specific activities of Sapp2p⁺⁸ and Sapp2p were therefore examined and compared using the Anson test, a spectrophotometric assay based on the digestion of hemoglobin. Purified Sapp1p isoenzyme was used as a positive control. Sapp2p activated by trypsin displayed 66% of the Sapp1p activity, whereas Sapp2p⁺⁸ displayed only 27%. The low activity of Sapp2p⁺⁸ compared to that of Sapp2p further supports the concept of assisted activation of pro-Sapp2p.

The Anson test was used to examine the effects of pH and temperature on the enzyme activity of Sapp2p activated by trypsin. Maximal activity of Sapp2p was observed at c. pH 4 and 37°C (Fig. 5). The dependence of Sapp2p activity on pH and temperature is rather sharp, especially when compared with Sapp1p, which is active over a wide range of conditions (Hrušková-Heidingsfeldová et al., 2001).

**Design of a fluorogenic substrate for Sapp2p activity testing**

As the peptides containing 4-nitrophenylalanine in the P₁′ position were not suitable for Sapp2p activity testing, cleavage of BSA by Sapp2p was examined in order to obtain information on the subsite preferences of the enzyme. BSA was cleaved into several fragments. Four of them were N-terminally sequenced, and scissile bonds were determined (Table 1). None of the four cleavage sites contained an aromatic residue in the P₁ or P₁₀ positions. By contrast, the P₁ position was always occupied by a polar amino acid. Aliphatic residues are preferred in the P₁₀ position. These observations correspond to the character of the pro-Sapp2p/ Sapp2p⁺⁸ processing site, where the hydrolysis occurs between asparagine and alanine. Altogether, the substrate specificity of Sapp2p appears to be not so broad as that of Sapp1p.

![Fig. 4. Processing of pro-Sapp2p by a membrane fraction of Candida parapsilosis. SDS-PAGE (a) and Western blot (b) analysis: unprocessed precursor (lane 1); trypsin-activated Sapp2p (lane 2); pro-Sapp2p processed by the membrane-bound proteinase (lane 3); a sample of Candida parapsilosis membrane fraction (lane 4).](https://example.com/fig4.png)
Based on these results, two fluorogenic peptides were designed, synthesized and tested as substrates of Sapp2p: Dabcyl-Pro-Lys-Val-Glu-Leu-Thr-Gly-Glu-EDANS and Dabcyl-Glu-His-Val-Lys-Leu-Val-Glu-EDANS. Both compounds were readily hydrolyzed by Sapp2p. However, the first peptide was cleaved at the same position (Glu/C3Leu), both by Sapp2p and by Sapp1p. This feature makes it less suitable for testing the presence of Sapp2p in the C. parapsilosis culture medium. The latter compound, by contrast, is cleaved differently by the Sapp isoenzymes (Fig. 6). The Lys/C3Leu bond is hydrolyzed by Sapp2p, and the Leu/C3Val bond is hydrolyzed by Sapp1p. This substrate was used to test the inhibition of Sapp2p by pepstatin A, a typical inhibitor of aspartic proteinases. Pepstatin A inhibited Sapp2p with $K_i = 0.6 \pm 0.021$ nM.

Discussion

Information on the SAPP2 gene product is scarce and contradictory. The only article describing the isolation of Sapp2p from the C. parapsilosis culture medium was published in 1993 by Fusek et al. (1993). They found that Sapp2p was present in a mixture with Sapp1p, and constituted only about 20% of the enzymes recovered from the culture supernatant. The low proportion of Sapp2p may be one of the reasons why it was not detected by others: the Sapp2p concentration might have been below the detection limits, particularly during the experiments with small volumes of the yeast culture.

Differences in the Sapp1p and Sapp2p substrate specificities might also have affected the search for Sapp2p in the C. parapsilosis culture supernatants. Fusek et al. (1993) observed inefficient cleavage of a synthetic peptide containing 4-nitrophenylalanine in the P1$^0$ position. However, their experiment employed only one substrate, and thus could not have clearly shown that chromogenic peptides with aromatic residues in P1 and P1$^0$ were less suitable for Sapp2p activity testing.

The present work proves that the SAPP2 gene product exhibits proteolytic activity. The design of the fluorogenic peptide that is readily cleaved by Sapp2p enables to establish assays for further characterization of this enzyme.

Sapp2p and Sapp1p differ also in their potential mode of activation. Both pro-Sapp1p and pro-Sapp2p were processed correctly by a membrane-bound Kex2-like proteinase in vitro. However, Sapp1p was also self-processed in acidic conditions, yielding a proteinase truncated by one amino acid at the N-terminus. Proteolytic activity of this truncated species did not differ from that of full-length authentic Sapp1p, which suggests that the autoactivation may be an alternative, redundant mechanism of Sapp1p zymogen processing (Dostál et al., 2005). By contrast, the pro-Sapp2p self-processing resulted in a protein extended by eight amino acids at the N-terminus, and the activity of this species was reduced in comparison with mature Sapp2p. This indicates that the self-processing does not play a role in Sapp2p activation. The differences in activation mechanisms and enzymological properties of Sapp1p and Sapp2p may reflect distinct roles that these isoenzymes probably play in C. parapsilosis.

Table 1. Hydrolysis of BSA by Sapp2p

<table>
<thead>
<tr>
<th>Fragment no.</th>
<th>Position of the scissile bond in the BSA sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$65 \times 66$</td>
<td>D E H V K* L V N E L</td>
</tr>
<tr>
<td>2</td>
<td>$69 \times 70$</td>
<td>K L V N E* L T E F A</td>
</tr>
<tr>
<td>3</td>
<td>$133 \times 134$</td>
<td>S H K D D* S P D L P</td>
</tr>
<tr>
<td>4</td>
<td>$206 \times 207$</td>
<td>L P K I E* T M R E K</td>
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Four BSA cleavage products were analyzed by N-terminal sequencing, and the scissile bonds were identified by comparing the results with the BSA sequence in the NCBI database. *cleavage site.
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Fig. 6. Cleavage of the fluorescent substrate by Sapp1p and Sapp2p. The analysis was performed using HPLC, a C-18 column and a linear gradient of methanol to water. The cleavage products were monitored using a fluorometric detector (excitation wavelength 360 nm; emission wavelength 485 nm). The sites hydrolyzed by Sapp1p and Sapp2p are indicated.

References


