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Regular paper

The action of ten secreted aspartic proteases of pathogenic yeast *Candida albicans* on major human salivary antimicrobial peptide, histatin 5*

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Candida albicans, **belonging to the most common fungal pathogens of humans, exploits many virulence factors to infect the host, of which the most important is a family of ten secreted aspartic proteases (Saps) that cleave numerous peptides and proteins, often deregulating the host's biochemical homeostasis. It was recently shown that *C. albicans* cells can inactivate histatin5 (His5), a salivary histidine-rich anticandidal peptide, through the hydrolytic action of Saps. However, the current data on this subject are incomplete as only four out of ten Saps have been studied with respect to hydrolytic processing of His5 (Sap2, Sap5, Sap9-10). The aim of the study was to investigate the action of all Saps on His5 and to characterize this process in terms of peptide chemistry. It was shown that His5 was degraded by seven out of ten Saps (Sap1-4, Sap7-9) over a broad range of pH. The cleavage rate decreased in an order of Sap2>Sap9>Sap3>Sap7> Sap4>Sap1>Sap8. The degradation profiles for Sap2 and Sap9 were similar to those previously reported; however, in contrast to the previous study, Sap10 was shown to be unable to cleave His5. On a long-time scale, the peptide was completely degraded and lost its antimicrobial potential but after a short period of Sap treatment several shorter peptides (His1-13, His1-17, His1-21) that still decreased fungal survival were released. The results, presented hereby, provide extended characteristics of the action of *C. albicans* extracellular proteases on His5. Our study contribute to deepening the knowledge on the interactions between fungal pathogens and the human host.**

Key words: secreted aspartic proteases, Sap, *Candida albicans*, antimicrobial peptides, histatin 5, candidiasis

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INTRODUCTION

The saliva plays a key role in the maintenance of a stable oral microflora, being a source of (glyco)proteins and peptides potentially serving as a growth substrate for microorganisms but also containing a wide variety of defensive systems that protect oral tissues against excessive microbial colonization (van't Hof *et al.*, 2014; Valentijn-Benz *et al.*, 2015). Ones of the the most abundant salivary components that possess potent antimicrobial activities are histatins, a family of low-molecular-weight, histidine-rich, cationic peptides produced and secreted by human parotid and submandibular-sublingual glands (Helmerhorst *et al.*, 2006). The family of histatins contains 12 members of which histatin 5 (His5), composed of 24 amino acid residues is one of the most prominent and the most potent with regard to antifungal activity, especially against *Candida albicans*, one of the most common fungal pathogen in humans. His5 is taken up by *C. albicans* cells through binding to cell wall proteins (Ssa1/2) and glycans. Once internalized, His5 inhibits the mitochondrial respiration and induces the formation of reactive oxygen species, causing the ultimate cell death by volume dysregulation and ion imbalance triggered by osmotic stress (Puri & Edgerton, 2014). Recent studies indicated that besides the antifungal activity His5 is able to reduce adhesion of *C. albicans* to epithelial cells (Moffa *et al.,* 2015).

C. albicans colonizes mucosal surfaces, including the oral cavity wherein it constitutes 40–60% of all yeast species present (Samaranayake *et al.*, 2009; Dynowska *et al.*, 2014). In healthy individuals this fungus is usually a harmless commensal. However, when the natural microflora of the oral cavity is imbalanced, owing to the reduced immunity, long-term use of antibiotics and drugs such as inhaled steroids, the use of dentures, immunosuppressive conditions such as in HIV patients, malignancies such as leukemia and nutritional deficiencies or age, the individual becomes at increased risk of infectious diseases such as candidiasis or thrush (Singh *et al.*, 2014). To remain a stable commensal organism or, in opposite, to become a causative agent of a serious disease, *C. albicans* defends itself against a multiplicity of innate immune mechanisms by activation of fungal virulence traits, among which the production of secreted aspartic proteases (Saps) plays a major role (Hube, 2001; Naglik *et al.*, 2004; Schaller *et al.*, 2005). Ten distinct Saps, similar in structure and function, of which eight (Sap1-8) are released into the extracellular space while Sap9 and Sap10 are glycosylphosphatidylinositol (GPI)-anchored to the cell membrane/wall, display a broad substrate specificity

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Abbreviations: AMP, antimicrobial peptide; CFU, colony-forming units; ESI, electrospray ionization; GPI, glycosylphosphatidylinositol; His5, histatin 5; HPLC, high pressure liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP-HPLC, reverse-phase HPLC; Sap, secreted aspartic protease; TFA, trifluoroacetic acid; YPD, yeast peptone dextrose.

at pH ranging from 2 to 7 (Aoki *et al.,* 2011). Thus, Saps are able to degrade a vast number of host's proteins such as salivary lactoferrin, α_2-macroglobulin, immunoglobulins, the proinflammatory cytokine interleukin-1β, cathepsin D, complement components, kininogens and precursors of several blood coagulation factors (Naglik *et al.*, 2003a; Schaller *et al.*, 2005; Bras *et al.*, 2012; Kozik *et al.*, 2015). Recent studies performed in our laboratory showed that Sap-catalyzed fragmentation of some proteins can result in the release of antimicrobial peptides (AMPs) such as hemoglobin-derived hemocidins (Bocheńska *et al.*, 2013), possibly to compete with other microorganisms in the same niche. On the other hand, we found that Saps have an ability to degrade some AMPs, such as LL-37 cathelicidin (Rapala-Kozik *et al.*, 2015) and kininogen-derived NAT26 and HKH20 peptides (Bochenska *et al.*, 2015) and deprive them of antifungal properties. Recently it was shown that *C. albicans* cells can inactivate His5 through the hydrolytic action of Saps (Meiller *et al.*, 2009). However, up to date, the data on the proteolytic processing of His5 by *C. albicans* enzymes are incomplete, because so far only four out of ten Saps have been studied with respect to this activity (Sap2, Sap5, Sap9-10). The aim of the current study was to investigate the action of all Saps on His5 and to characterize this process in terms of peptide chemistry.

MATERIALS AND METHODS

Materials. His5 (DSHAKRHHGYKRKFHEKHHSHRGY) peptide was purchased from Eurogentec (Liege, Belgium). Unless otherwise stated, all other chemicals and materials used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Yeast strain and culturing. The *C. albicans* ATCC 10231 strain from the American Type Culture Collection (Manassas, VA, USA) was cultured overnight at 30°C in YPD medium containing 0.5% yeast extract, 1% peptone, and 2% glucose.

Production and purification of Sap1–10 enzymes. Ten Sap isozymes were overproduced in *Pichia pastoris* expression system (Invitrogen, Carlsbad, CA, USA) and purified according to the published method (Aoki *et al.*, 2011) with slight modifications, described elsewhere (Rapala-Kozik *et al.*, 2015). The high purity of the obtained proteins (>95% for each Sap) was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system (Laemmli, 1970). Protein concentrations were determined by the Bradford assay (Bradford, 1976). The proteolytic activity of all Saps was measured using BODIPY FL casein substrate (Invitrogen) at the optimal pH for each Sap.

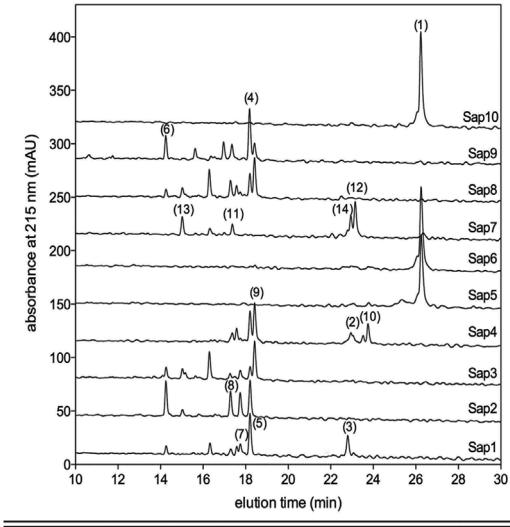
Analysis of the Sap-dependent cleavage of His5. Individual Saps were found to differ markedly in the ability to hydrolyze His5. To make their effects on His5 measurable, we accordingly adjusted the enzyme:substrate ratio (range of 1:50–1:10 000) or/and the incubation time (0 h, 0.25 h, 0.5 h, 1 h, 2 h or 5 h). His5 was used at 50 μM concentration in 10 mM acetate buffer at pH 4 (for Sap2, Sap3 and Sap8), pH 5 (optimal for Sap1, Sap4–6 and Sap9) or in 10 mM phosphate buffer at pH 6 (optimal for Sap7 and Sap10) at 37°C. After incubation, the reactions were stopped by adding pepstatin A (to a final concentration of 10 μM) and placed on ice for 15 min. The samples were (i) tested for antifungal activity, and (ii) additionally mixed with 0.17 M HCl and analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC).

RP-HPLC analysis of His5 cleavage by Sap1–10. Sap-catalyzed cleavage of His5 was analyzed by HPLC using a Dionex Ultimate 3000 system (Thermo Scientific, Waltham, MA, USA). The peptides were separated on a Eurosil Bioselect 300-5 C-18 column (5 μm, 4 mm × 250 mm) equipped with a precolumn (both from Knauer, Berlin, Germany) in a two-solvent system (solvent A: 0.1% TFA in water; solvent B: 0.08% TFA in 80% acetonitrile [Merck, Darmstadt, Germany]), at a flow rate of 1 ml/min, with a spectrophotometric detection at 215 nm. The gradient of 0–25% solvent B over 31 min was applied for the separations of His5 fragments. Fractions were collected, evaporated to dryness, and analyzed by mass spectrometry (MS).

Identification of His5 fragments by mass spectrometry. Evaporated fractions obtained from RP-HPLC separations were resuspended in 30% methanol with 0.1% HCOOH and analyzed using an HCTultra ETDII mass spectrometer (Bruker, Bremen, Germany). Samples were directly injected with a syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of 180 μl/h to an electrospray ionization (ESI) ion source, operated in positive ion mode at a capillary voltage of 3.5 kV, nebulizer pressure of 10 psi, drying gas flow of 5 l/min, and ion source temperature of 300°C. The ion trap analyzer of the spectrometer was set at both MS and MS/MS (tandem mass spectrometry) mode. The peptide identification was performed both manually and automatically, using DataAnalysis™ 4.0 software, Biotools™ 3.2 software (Bruker), and an in-house Mascot server, ver. 2.3.0 (Matrix Science, UK) (Perkins *et al.,* 1999), that searched against the Swiss-Prot database.

Antifungal activity assays – a viability test and a membrane permeabilization assay. *C. albicans* cultures were harvested, washed three times with 10 mM phosphate buffer (pH 7.4) with 5 mM glucose, counted with use of Burker chamber and diluted to a final density of 2×10^6 colony-forming units (CFU)/ml. The assays were used for the determination of the candidacidal activities of the AMPs. For viability assay, 5×10^3 yeast cells were incubated for 2 h at 37°C with Sap-cleaved His5 (at a final concentration of 25 μM) or peptides collected and evaporated from HPLC analyses in 10 mM phosphate buffer (pH 7.4) with 5 mM glucose, which stabilized the yeast growth. After that time, serial dilutions of the incubation mixtures were plated on YPD agar. After a 24-h incubation at 30°C, the numbers of CFU were counted. The percentage of killed cells was calculated relative to control microorganisms incubated in the absence of AMPs. The measurements were performed in triplicates. Statistical analysis was performed using the Kruskal-Wallis test (included in the GraphPad Prism 6.0 software) at the significance level of $P<0.05$.

Fungal membrane permeabilization was determined using the fluorescent dye SytoxGreen (Molecular Probes, Eugene, OR, USA). The assay mixture contained a greater number of yeast cells (5×10^4) compared with viability assay in order to visualize cell permeation more easily. After 2-h incubation at 37°C, 50 μl of mixtures were transferred to 96-well plate and 0.2 μM of SytoxGreen was added. Stained yeast cells were quantified by the observation under a fluorescence microscope Nikon Eclipse Ti (Nikon, Tokyo, Japan) with the filters set at an excitation wavelength of 465–495 nm and emission at 515–555 nm. The percentage of permeabilized cells was a ratio of the number of green-fluorescent cells to the total number of cells counted.



No.	Retention time [min]	Sequence	Determined molecular mass [Da]	Produced by Saps
1	26.5	**DSHAKRHHGYKRKFHEKHHSHRGY**	3036.3	-
2	23.4	**DSHAKRHHGYKRKFHEK**	2161.4	Sap1,2,3,4,9
3	23.2	AKRHHGYKRKFHEK	1821.0	Sap1
4	18.6	HHSHRGY	891.4	Sap1,2,3,4,8,9
5	18.2	AKRHHGY	867.5	Sap1
6	14.7	FHEK	559.6	Sap1,2,3,9
7	18	KRKFHEK	971.6	Sap1
8	17.3	**DSHAKRHHGYKRK**	1618.9	Sap2,3,9
9	18.4	DSHAKRHHGY	1206.6	Sap2,3,4,8
10	23.9	KRKFHEKHHSHRGY	1847.1	Sap4
11	17.7	DSHAKRHHGYKR	1490.8	Sap4,7
12	23.4	**DSHAKRHHGYKRKFHEKHHSH**	2659.9	Sap7
13	15.3	RGY	394.2	Sap7
14	23	FHEKHHSHRGY	1433.7	Sap3,7,8

Figure 1. HPLC profiles of His5 samples treated with Saps. His5 (50 μM) was digested with the 10 recombinant Saps at an enzyme:substrate molar ratio of 1:50 in 10 mM buffer at the optimal pH for each enzyme at 37°C for 5 h. Representative chromatograms show the ability of seven different Saps to hydrolyze His5. Peptides are annotated with numbers corresponding to the MS/MS-identified amino acid sequences specified beneath the chromatograms.

RESULTS

Susceptibility of synthetic His5 peptide to Sap-catalyzed cleavage and inactivation

Representative chromatograms, obtained from RP-HPLC analysis of His5 samples after 5-h digestions with recombinant Saps at the optimal pH for each individual enzyme and at a 1:50 enzyme:substrate ratio are presented in Fig. 1. This AMP was effectively cleaved by Sap1-4 and Sap7-9. The sequences of all major obtained products were determined by MS/MS and are listed in Fig. 1. Simultaneously conducted viability assay showed that the Sap-dependent degradation of His5 deprived this peptide of antifungal activity (Fig. 2).

The time course of Sap-dependent cleavage of His5 correlates with the decrease in its antifungal activity

Further experiments were aimed at determining the kinetic characteristics of Sap-dependent His5 cleavage and of the associated decay in the antifungal activity of this AMP. Additionally, the molar enzyme:substrate ratio was varied according to the previous estimation of the relative cleavage ability of individual Saps. Results are presented in Fig. 3. The cleavage rate decreased in

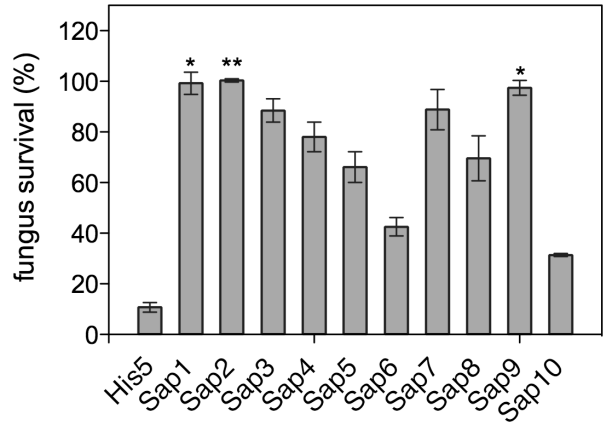


Figure 2. Antifungal activities of Sap-treated His5 samples. Samples of His5 with Saps, corresponding to those analyzed by HPLC and characterized in Fig. 1, were tested for killing activity against *C. albicans* (5×10^3 CFU). Numbers of viable colonies on agar plates are expressed relative to that of yeast cells not treated with His5. Data bars showing the relationship between the loss of antifungal properties and the Sap-capability to degrade His5, represent mean values from three determinations ± standard deviation. Asterisks denote the statistical significance at $P<0.01$ (**) or $P<0.05$ (*).

an order of Sap2 > Sap9 > Sap3 > Sap7 > Sap4 > Sap1 > Sap8. The first cleavage for five out of seven Saps—Sap1-4 (Fig. 3A–D) and Sap9 (Fig. 3G) – was between residues Lys17 and His18 giving DSHAKRHHGYKRKFHEK (2) and HHSHRGY (4) peptides. Simultaneously, DSHAKRHHGYKRK (8) peptide was formed by Sap2-3 and Sap9, and DSHAKRHHGY (9) as well as KRKFHEKHHSHRGY (10) by Sap4. Further fragmentation by Sap1 resulted in AKRHHGYKRKFHEK (3) and AKRHHGY (5) products, by Sap2 and Sap9 in FHEK (6) and additionally by Sap2 in DSHAKRHHGY (9) peptide, whereas further hydrolysis by Sap3 produced DSHAKRHHGY (9) and KRKFHEK (7) peptides. The time course of His5 degradation by Sap7 (Fig. 3E) was essentially different. The cleavage of His5 by Sap7 resulted mainly in the appearance of long DSHAKRHHGYKRKFHEKHHSH (12) peptide which after 1 h was slowly proceeded to DSHAKRHHGYKR (11) product. The hydrolysis of His5 by Sap8 (Fig. 3F) caused production of DSHAKRHHGY (9), HHSHRGY (4) and FHEKHHSHRGY (14) peptides. Although addition of Saps decreased antifungal activity of His5 itself, with exception of Sap8 (Fig. 4B), the complete loss of antifungal activity appeared after 1 h, suggesting that some peptides can still possess antifungal activities. For Sap9, for instance, the antifungal activity was maintained at a constant level at every measured time.

Fragments of His5 that still possess antifungal activity

In order to check which His5-derived fragments are responsible for sustained antifungal activity, fractions purified with HPLC method were subjected to two different survival assays. Out of fragments tested, three peptides, His1–21 (12), His1–17 (2) and His1–13 (8) were shown to possess the antifungal activities (Fig. 5). His1–21 and His1–17 efficiently inactivated fungal cells at the peptide concentration similar to that effective for His5 action, whereas His1–13 was able to decrease fungal survival to about 50% when incubated with 5000 cells. Increasing the number of cells ten-fold in the fluorescent

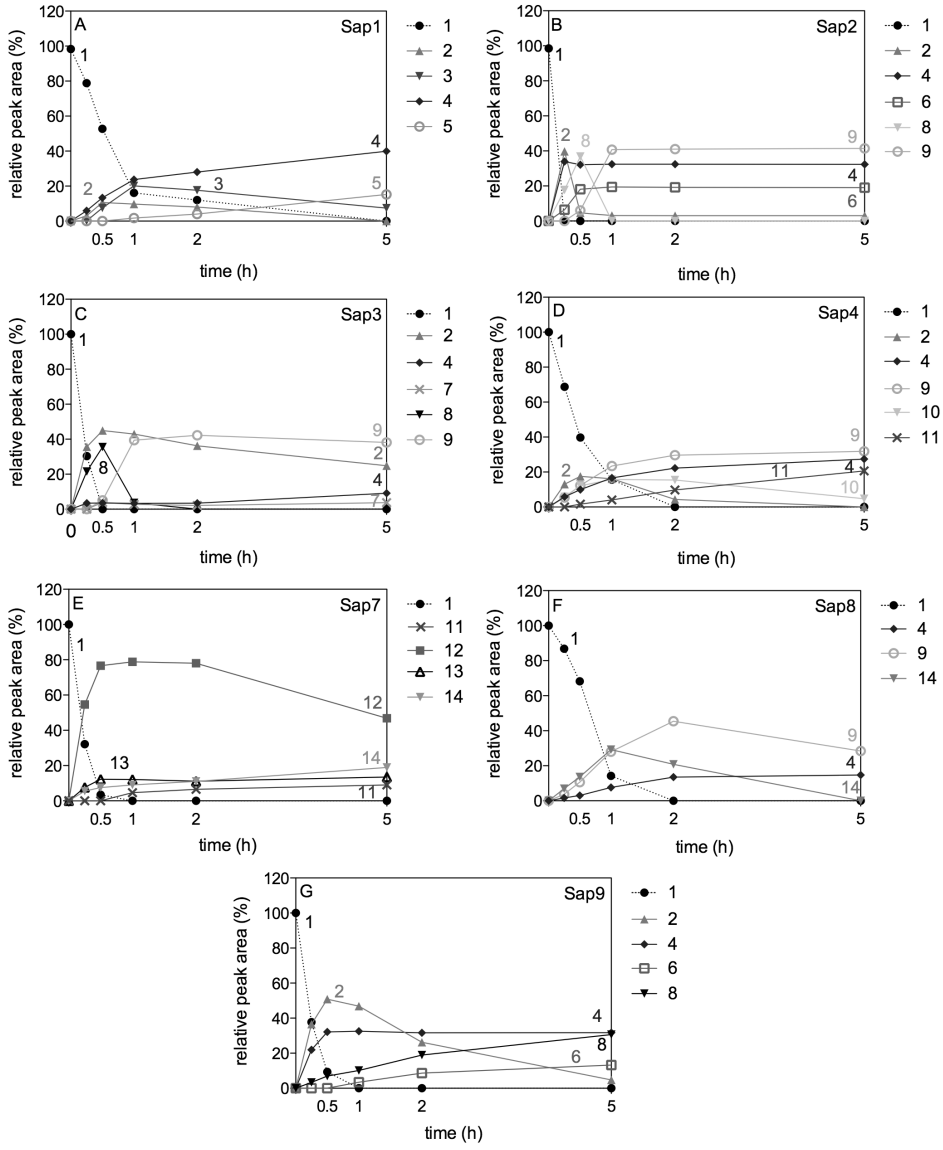


Figure 3. Time course of the cleavage of His5 by Saps.
His5 (50 μM) was incubated with Saps for various times (0–5 h) at enzyme:substrate ratios different for each Sap: 1:50 for Sap1 (**A**), 1:10000 for Sap2 (**B**), 1:1000 for Sap3 (**C**), 1:100 for Sap4 (**D**), 1:100 for Sap7 (**E**), 1:50 for Sap8 (**F**) and 1:5000 for Sap9 (**G**). The peak areas obtained from chromatographic data are expressed relative to that of the substrate at the beginning of the reaction. Only peaks with a relative area of at least 10% were taken into account and annotated with numbers corresponding to the sequences (determined by MS/MS) specified in Fig. 1.

assay slightly enhanced the survival of *C. albicans*, but the relative antifungal ability of tested peptides remained unchanged.

The pH dependence of His5 degradation by Saps

Analysis of the pH dependence of His5 degradation by Saps (Fig. 6) was performed at pH 4–7, at the same molar ratio of enzyme:substrate as used in the kinetic studies presented above, for time sufficient for tracking the disappearance of the substrate (15 min for Sap2, 30 min for Sap3 and 1 h for Sap1, Sap4, Sap7–9). These experiments showed that, despite that Saps display the highest hydrolytic activity at acidic conditions, most proteases tested in this study, with exception of Sap8, were active at pH between 6–7, i.e., optimal pH of the saliva. The highest activity at pH 7 was presented by Sap3, Sap4 and Sap9.

DISCUSSION

C. albicans, being one of the most common pathogen of humans and invading different niches of the organism, including mucosal surface within the oral cavity, exploits several virulence factors to infect the host's tissues and avoid the host's defence mechanisms. One of the most potent and best-studied virulence mechanisms is production of ten different Saps with broad substrate specifity, which can degrade host cell surface molecules and attack cells and molecules of the host immune system to avoid or resist antimicrobial activity (Naglik *et al.,* 2003a; Schaller *et al.,* 2005).

The oral cavity is one of the most heavily microbially colonized parts of our body. The importance of saliva in the maintenance of oral health is well recognized, especially in patients suffering from a reduced salivary output who are much more susceptible to fungal infections than

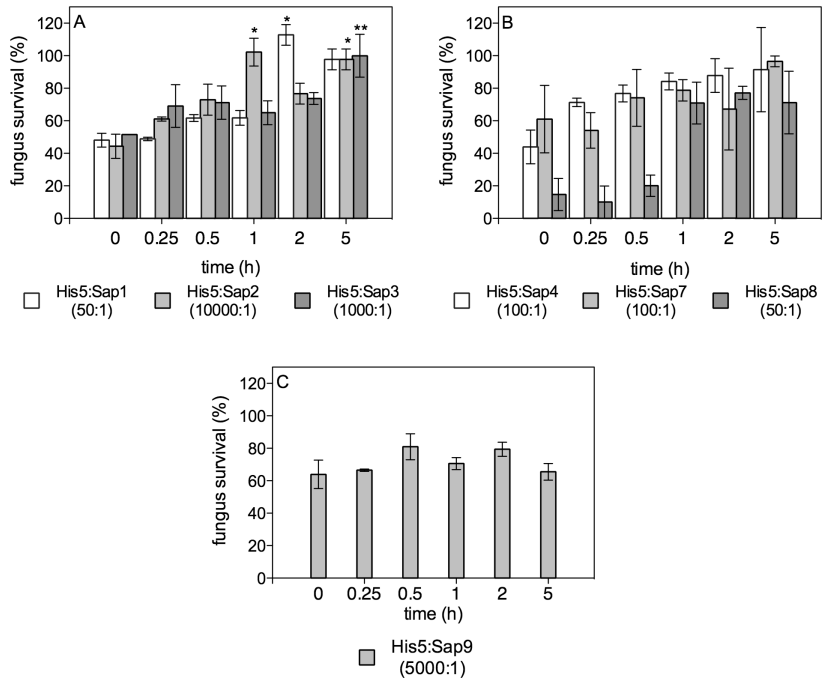


Figure 4. The antifungal activity of Sap-treated His5 samples from kinetic analysis.
His5 (50 μM) was incubated with Saps for various times (0–5 h) at enzyme:substrate ratio different for each Sap as specified in legends. The numbers of viable colonies on agar plates for samples of Sap-treated His5 are expressed relative to that of yeast cells not treated with this AMP. Data bars represent mean values ± standard deviation (three determinations). Asterisks denote the statistical significance at $P<0.01$ (**) or $P<0.05$ (*).

healthy individuals (van't Hof *et al.*, 2014). His5 is the potent antimicrobial peptide responsible for protection of oral mucosa. Recently it was shown that *C. albicans* is capable of growing in human saliva (Valentijn-Benz *et al.*, 2015). When the energy metabolism is suppressed, the yeast's resistance against AMPs such as histatins is enhanced (Veerman *et al.*, 2007). Additionally, in the population most vulnerable to oral candidiasis, i.e., HIV patients, an increased growth of *C. albicans* was correlated with decreased levels of His5 (Torres *et al.,* 2008). In a previous study (Meiller *et al.,* 2009) it was shown that *C. albicans* cells can degrade His5 *in vitro*. Based on comparisons between *C. albicans* wild-type strain and Sap-null mutants, the degradation ability was attributed to Sap proteases. Investigation of four proteases, Sap2, Sap5, Sap9 and Sap10, in terms of His5 fragmentation revealed that Sap5 is ineffective in His5 cleavage and the main protease responsible for His5 degradation is Sap9. In the present study, we attempted to expand the characteristics of His5 degradation on all ten *C. albicans* proteases, to investigate this aspect of the dynamic host-pathogen interplay during development of oral candidiasis more deeply. We confirmed that Sap5 was unable to degrade the AMP tested and that the degradation profiles for Sap2 and Sap9 were similar to those previously reported. However, in contrast to that previous work, Sap10 was not able to cleave His5. At present, no reason can be provided to explain this discrepancy.

The key finding presented in this paper is that seven out of ten *C. albicans* Sap family members (Sap1–4 and Sap7–9, used as purified recombinant proteins) can rapidly degrade salivary AMP, His5. The observed cleavage patterns are consistent with previous reports of Sap specificities (Koelsch *et al.*, 2000; Albrecht *et al.,* 2006; Aoki *et al.,* 2011; Schild *et al.,* 2011). While Sap1–6 and Sap8 have broad substrate specificity and prefer to cleave peptide bonds at positively charged or large hydrophobic amino acids, such as Arg, Lys, Leu, Phe, and Tyr, Sap7 and Sap9–10 have narrower substrate specificities and prefer amino acids such as Met, Arg and His. These specificity characteristics explain the formation of the same first produc – DSHAKRHHGYKRKFHEK – by five out of seven Saps from synthetic His5, and its derivatives cleaved after Lys or Tyr after longer period of time. They also explain, rarely occurring among proteases, preferred cleavage after histidine residues for Sap7 and after Arg afterwards. The ability of Sap9 to degrade His5 only after Lys is consistent with the confirmed preference of this protease for cleavage of peptide bonds after basic residues.

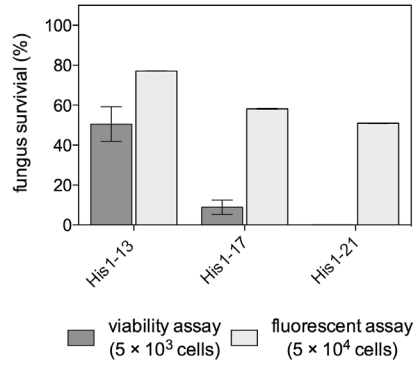


Figure 5. Cleavage fragments derived from His5, still possessing antifungal activity.
The peptides purified with HPLC method were incubated with different amount of *C. albicans* cells at 37°C for 2 h. The percent of *C. albicans* survival in viability assay represents the number of viable colonies on agar plates expressed relative to yeast cells not treated with this AMP and in fluorescent assay the ratio of dead to living yeast cells stained with SytoxGreen. Data bars represent mean values from three determinations ± standard deviation.

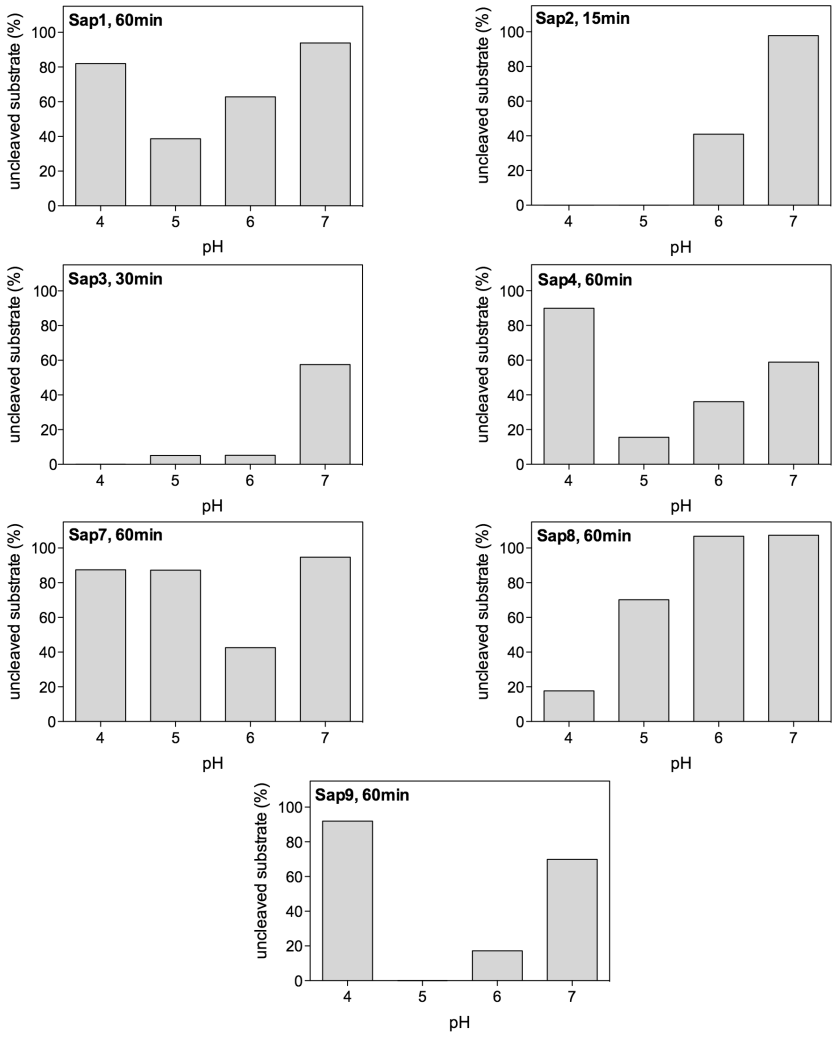


Figure 6. pH–dependence of the degradation of His5 by Saps.
Bars represent the percents of remaining His5 (5 μM) separated by RP-HPLC after Sap-treatment at variable enzyme:substrate molar ratio, chosen as sufficient for the activity of each Sap (Fig. 3), at pH within a range between 4 to 7 at 37°C for the time specified on graphs.

The hydrolytic cleavage of His5 correlates with the loss of antifungal activity of this peptide. After long time of incubation with Saps, short His5 derivatives appeared and the antimicrobial potency against *C. albicans* was completely eliminated. However, in the early degradation mixture, antifungal activity was sustained. The reason for that is the production of several shorter peptides such as His1–13, His1–17 and His1–21 that still possess some antifungal activity. As reported in previous studies (Raj *et al.,* 1990; Xu *et al*., 1993; Rothstein *et al.,* 2001; Helmerhorst *et al*., 2006) there is a large range of His5 derivatives which are able to kill *C. albicans*. These derivatives are the result of His5 degradation by salivary proteases. One of the peptides which occurs due to salivary degradation is His1–17 whose antifungal action was previously documented (Helmerhorst *et al*., 2006). Taking into consideration, that His1–21 is the longest of the peptides described and that all, investigated till present, His5-derivatives containing more than 16 amino acid residues maintain their anticandidal activity, there is no surprise that His1–21 is also antimicrobial. Although His1–13, also produced in saliva, was previously not confirmed to be antimicrobial, our studies showed that it can partly decrease fungal survival. However, it can be not sufficient enough to fully kill *C. albicans*, as it is only a part of the smallest His5-derivative peptide with conformed antimicrobial properties – AKRHHGYKRKFH. The latter peptide is also the only one from His5 derivatives which currently is in phase I/II and IIb clinical trials and presents the safety, tolerance and efficacy in treatment of oral *Candida* infections (http://www.demegen.com/articles/P-113-Candidiasis-results.htm).

The analysis of pH dependence of His5 inactivation by Saps demonstrated that most of the tested proteases, with exception of Sap8, have the ability to degrade AMP in the oral cavity, where normal pH of saliva is within a range of 6.2–7.6 (Surdacka *et al.,* 2007; Baliga *et al.,* 2013). Regardless of the fact that these enzymes are more active in acidic conditions, the diversity and the broad range of pH (2–7) optimal for the enzymatic activity, allows these *C. albicans* enzymes to target substrates in different niches of the host organism.

Our current findings corroborate the study of Meiller and coworkers (2009) and also indicate that among the ten members of the Sap family, Sap9 protease is the most effective isoenzyme responsible for His5 degradation. It is the enzyme attached to the cell membrane/wall through GPI-anchor, crucial for infection process (Albrecht *et al.,* 2006) and most active in the oral environment. *SAP9* gene is one of the most highly ex-

pressed protease genes *in vivo* during human mucosal infections, in oral reconstituted human epithelium (RHE) models (Naglik *et al.*, 2008). In addition, it is frequently expressed by the yeast both in the commensal stage and during infection (Albrecht *et al.,* 2006). Besides Sap9, also soluble Saps can be relevant, especially Sap3 and Sap4 as they showed His5-degrading ability at pH 6-7. *In vivo* analysis of the expression of *C. albicans SAP1-SAP8* genes in oral candidiasis (Schaller *et al.*, 2001; Naglik *et al.,*1999; 2003b) indicated the highest frequency of *SAP2* expression for both colonized and infected patients. Considering the proteases which were hereby found to degrade His5, *SAP1*, *SAP2*, *SAP4*, *SAP7* and *SAP8* genes are expressed at a significant level during oral infections (Naglik *et al.,* 2003b). *SAP1-3* genes, expressed during mucosal infections (Naglik *et al.*, 1999, 2008) are significant for infection process in general, whereas *SAP4* belonging to *SAP4-6* subfamily is essential during hyphal invasion (Schaller *et al.*, 2001; Silva *et al.,* 2014). Moreover, the clinical specimen obtained from 29-year old female patient suffering from acute oral candidosis and from a lesion of chronic oral candidosis in an HIV-infected patient showed the presence of SAP1-, 3- and 6-encoding genes in case of the first patient and *SAP2* expression in the latter case (Schaller *et al.*, 1998).

To conclude, in the present study we showed that most of aspartic peptidases, secreted by *C. albicans* are able to degrade salivary antimicrobial peptide His5; however, during initial fragmentation, His5 derivatives which are still able to reduce the survival of this fungus appear.

Conflicts of interests

The authors declare that they have no competing interests.

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