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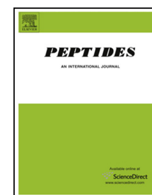

Secreted aspartic peptidases of *Candida albicans* liberate bactericidal hemocidins from human hemoglobin

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ABSTRACT

Secreted aspartic peptidases (Saps) are a group of ten acidic hydrolases considered as key virulence factors of *Candida albicans*. These enzymes supply the fungus with nutrient amino acids as well as are able to degrade the selected host's proteins involved in the immune defense. Our previous studies showed that the human menstrual discharge is exceptionally rich in bactericidal hemoglobin (Hb) fragments – hemocidins. However, to date, the genesis of such peptides is unclear. The presented study demonstrates that the action of *C. albicans* isozymes Sap1–Sap6, Sap8 and Sap9, but not Sap7 and Sap10, toward human hemoglobin leads to limited proteolysis of this protein and generates a variety of antimicrobial hemocidins. We have identified these peptides and checked their activity against selected microorganisms representative for human vagina. We have also demonstrated that the process of Hb hydrolysis is most effective at pH 4.0, characteristic for vagina, and the liberated peptides showed pronounced killing activity toward *Lactobacillus acidophilus*, and to a lower degree, *Escherichia coli*. However, only a very weak activity toward *Staphylococcus aureus* and *C. albicans* was noticed. These findings provide interesting new insights into pathophysiology of human vaginal candidiasis and suggest that *C. albicans* may be able to compete with the other microorganisms of the same physiological niche using the microbicidal peptides generated from the host protein.

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1. Introduction

Hemoglobin (Hb) is a precursor of plethora different biologically active peptides which emerge from this protein after a limited proteolysis [18,21,38]. Hb has been even proposed as the model protein which serves as a proteinaceous substrate for generation of tissue-specific pool of bioactive peptides [18,20]. Many of these Hb fragments possess also a microbicidal activity and for such specific family of peptides a common name "hemocidins" has been coined [26,28]. This quite heterogenous group can be effectively obtained *in vitro*, by enzymatic or chemical fragmentation of Hb from many vertebrate species [2,7,8,10,15,23,29,37,39]. Studies using circular dichroism (CD) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, liposomes, planar lipid bilayers and membrane-mimicking sodium dodecyl sulphate (SDS) micelles suggest that the mechanism of action of these peptides is related to disruption of the plasma membrane of microorganisms by detergent-like or, so called, "carpet-like" manner [5,25,27,46]. In aqueous solutions, hemocidins are randomly structured but after electrostatically driven contact with membranes or with membrane-mimicking agents they reconstruct the structural alpha-helical arrangement, typical for the corresponding parts of the maternal protein, followed by incorporation into the lipid bilayers and destabilization of them, causing finally the lysis of microbial cells.

To date, *in vivo*, hemocidins have been ascertained in tick guts [5,13,36], in epithelium and liver of fishes [47,52], in rabbit vaginal fluid [40], in human placenta [23], in human post-partum uterine fluid [28] as well as in human menstrual discharge [30,49]. Among such examples, the menstrual hemocidins appear as the most interesting ones because they constitute a new, previously unknown mechanism which periodically, during physiological menstrual bleedings, regulates the number and the composition of the human vaginal microflora. The bactericidal Hb-derived peptides have been found as the main low-molecular mass polypeptide compounds of the normal menstrual discharge and, simultaneously, they have also been found as the exceptionally diverse group – the peptide maps obtained from the discharge of three different healthy women varied significantly from each other [29]. Moreover, differences

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have also been demonstrated between the peptide maps obtained from menstrual discharges of the same woman but collected during three successive monthly bleedings. These facts suggest that the generation of such peptides in vagina has rather a random character and reflects the complexity and diversity of the natural and individual vaginal ecosystem.

Menstrual hemocidins demonstrated a pronounced activity toward Gram-negative bacteria, especially *E. coli*, and such fact inspired us to hypothesize that the frequent vaginoses and colonization of vagina by such particular bacterium in postmenopausal women might be related with the decay of menstrual bleedings [30]. Moreover, also the length of monthly menstrual bleeding and, simultaneously, the length of exposition of vagina to bactericidal hemocidins appear to be a significant factor. This has been demonstrated during the statistical comparison of an average declared length of the menstrual bleeding period of women with the symptoms of urogenital infections with the declared length of bleeding for the group of control, healthy women. The last group presented statistically longer period of menstrual bleedings [49].

However, the process of generation of menstrual hemocidins is, to date, unclear. Menstruation comprises disintegration and then removal of the outer layer of epithelium at the end of the secretory phase of the non-pregnant cycle. Excreted fluid comprises tissue remains, mucus, blood plasma, proteolytic enzymes and red blood cells. The plasminogen activators produced by late secretory and menstrual endometrium prevent this mixture from clotting [14]. Disintegration of tissues is driven by numerous proteolytic enzymes such as matrix metallopeptidases, matrix cathepsin D and leukocyte serine peptidases [4,33,35]. One can speculate that these enzymes are also responsible for limited proteolysis of Hb liberated from erythrocytes, which undergo the lysis in an acidic environment of vagina. However, such hypothesis still needs experimental verification. In the present paper we demonstrate that microbicidal hemocidins can be effectively generated from Hb by quite different factors – Saps – the specialized secreted acid-like aspartic peptidases of *C. albicans* [3,31,32,42,51]. This fungus is a principal factor causing very common vaginal candidiases [1,12,17] and Saps are important virulence factors of mentioned commensal microorganism, allowing to successfully colonize the host, especially immune-compromised patients. The presented results prove that bactericidal hemocidins generated by Saps from human Hb are also able to regulate the composition and the number of microorganisms which compete with *Candida* in the same physiological niche – especially vagina. Moreover, the work provides some interesting new insights into the knowledge about the specificity of Sap's action.

2. Materials and methods

2.1. Chemicals and materials

If not otherwise stated, all chemicals and materials used in this study were purchased from Sigma, USA.

2.2. Microbial strains and culture conditions

Escherichia coli K12 ATCC 10798 was maintained in Luria Broth (LB), *S. aureus* ATCC 25923 was maintained in Tryptic Soy Broth (TSB), *C. albicans* ATCC 10231 was grown in Yeast Extract-Peptone-Dextrose (YPD) broth and *Lactobacillus acidophilus* ATCC 4356 was grown in de Man-Rogosa-Sharpe (MRS) broth. All microorganisms were cultivated on a horizontal shaker; *E. coli*, *S. aureus* and *L. acidophilus* at 37 °C while *C. albicans* at 30 °C. *E. coli*, *S. aureus* and *C. albicans* were grown in aerobic conditions whereas *L. acidophilus* was cultivated in anaerobic atmosphere using a MG500 workstation (Don Whitley Scientific, United Kingdom).

2.3. Antimicrobial assays

Determination of microbicidal activity was performed using fresh, mid-logarithmic phase microorganism cultures prepared as 1% (v/v) inocula from the overnight ones. Obtained cultures were washed twice in 10 mM sodium phosphate buffer pH 6.8 and diluted in this buffer to 2×10^5 CFU/10 μl. These suspensions were preincubated with the equal volumes of peptide solutions, diluted serially, plated on the appropriate medium solidified with agar and then incubated overnight. Obtained colonies were counted and the percent of killed cells was calculated in relation to the control microorganisms incubated without peptides.

2.4. Purification of human Hb

Hb was purified from a freshly-made water lysate of human erythrocytes using an anion-exchange chromatography on a Mono Q HR 10/10 column (GE Healthcare, USA), equilibrated in 20 mM Tris–HCl pH 8.0 and eluted using a linear gradient up to 0.15 M NaCl in the same buffer. Collected fraction of Hb was desalted and concentrated on a 10 kDa molecular weight cut-off membrane, and then quantified spectrophotometrically according to the molecular absorption coefficient from [11].

2.5. Production and purification of recombinant Saps

All 10 Sap isozymes were expressed in *Pichia pastoris* GS115 (Invitrogen, USA) as described previously [3]. Instead of an affinity chromatography, in the final purification step the anion-exchange chromatography on a Mono Q HR 10/10 column (GE Healthcare, USA) was performed. The column was equilibrated in 20 mM Tris–HCl pH 7.5 and eluted using a linear gradient up to 0.3 M NaCl in the buffer as above. The proteolytic activity of all purified isozymes was confirmed using BODIPY FL casein substrate (Invitrogen, USA) hydrolyzed in 0.1 M buffer at pH corresponding to the highest activity for each SAP enzyme (citrate 3.0–4.0, acetate 5.0 or phosphate 6.0–6.5). Decomposition of such substrate was assessed fluorimetrically at 485/530 nm excitation/emission wavelengths. The concentration of enzymes was determined by a Bradford assay while their homogeneity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The identity of the enzymes was confirmed by N-terminal sequencing of protein bands electrotransferred from the SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane.

2.6. Digestion of Hb by Saps

Hb was digested by Saps for 3 or 18 h at 37 °C in 0.1 M sodium citrate buffer pH 3.0, 4.0, 5.0 or 6.0 using 50: 1 substrate to enzyme weight ratio. After digestion the reaction mixtures were acidified by trifluoroacetic acid (TFA) to pH 2.0, spun down for 5 min at 16,000 g and filtered through a 0.45 μm filter.

2.7. Chromatographic separations

High-pressure liquid chromatography (HPLC) was performed using Dionex (USA) P680 system and Discovery BIO Wide Pore C18 4.6 mm × 250 mm column (Supelco, USA). Two buffers were used, A – 0.1% (v/v) trifluoroacetic acid (TFA), B – 0.07% TFA, 80% (v/v) acetonitrile, with the flow rate of 1 ml/min and spectrophotometric detection at 220 nm. Two different linear gradients were used: the fast one, 5–100% B in 10 min, and the slow one, 15–80% B in 25 min.

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