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ROLE OF ASPARTIC PROTEINASES IN *CANDIDA ALBICANS* VIRULENCE. PART II: EXPRESSION OF *SAP1-10* ASPARTIC PROTEINASE DURING *CANDIDA ALBICANS* INFECTIONS *IN VIVO*

ROLA PROTEAZY ASPARTYLOWEJ W WIRULENCJI *CANDIDA ALBICANS* CZĘŚĆ II: EKSPRESJA *SAP1-10* PROTEAZY ASPARTYLOWEJ PODCZAS ZAKAŻEŃ *CANDIDA ALBICANS IN VIVO*

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Abstract: *Candida albicans* is an opportunistic fungal pathogen known to produce several secreted hydrolytic enzymes, among which aspartic proteinases are considered to be a key virulence factor in pathogenesis. During last decade, Saps have been extensively studied in several *in vivo* studies based on human samples and animal models. It has been demonstrated that *SAP*5 and *SAP*9 are the most highly expressed proteinase genes *in vivo*. Despite many studies, very little is known about *SAP*7 and *SAP*8 role in *C. albicans* pathogenesis. Moreover, this review presents Sap regulation by nutritional supplementation and environmental factors, i.e. temperature, pH and the growth phase of *C. albicans* cells. In addition, Saps presence is discussed in *Candida tropicalis* as well as *Candida parapsilosis* and *Candida guilliermondii* as contribution of these non-*albicans Candida* strains in clinical infections is gradually increasing. Furthermore, the review underscores the need for studies using Sap enzymes as a potential drug-target due to their key role in virulence of *Candida* spp. The studies using the classical aspartic PI pepstatin A and HIV PIs provided evidence for the contribution of Sap to *C. albicans* virulence. Therefore, more conclusive studies concerning the 10 *SAP* gene expression and their regulation during infective process, association of Saps production with other virulence processes of *C. albicans* and Saps immune response in animal and human infection still have to be conducted.

Słowa kluczowe: *Candida albicans,* kandydoza, proteaza aspartylowa, wirulencja
Key words: *Candida albicans,* aspartic proteinases, candidiasis, virulence

1. Aspartic proteinase genes expression during *Candida albicans* infections *in vivo*

Enzymatic activities of *C. albicans* have received considerable attention in several *in vivo* studies [7, 15, 36, 42, 44]. Thus, the role of Saps in the development and progression of candidiasis has been studied for systemic and mucosal candidal infections [4, 23, 31, 37, 43, 53]. In addition, the research involving human samples and animal models are discussed in the manuscript (Table I). With regard to human mucosal infections, an early study by Schaller et al. [43] suggested pathogenetic role of the Sap1-3 during oral candidiasis. This notion was supported when a sensitive, RT-PCR technique was used that was able to detect *SAP*1 to *SAP*3 transcripts in patients with oral candidiasis [34]. On the contrary, Hube and Naglik [22] demonstrated the production of Sap1-8 proteinases during oral and vaginal candidiasis. The results indicated that *SAP*1, *SAP*3, *SAP*4, *SAP*7, *SAP*8 expression was correlated with oral disease, whereas *SAP*1, *SAP*3, and *SAP*6-8 expression was correlated with vaginal disease. It should be noted that although *SAP*1, *SAP*3, and *SAP*8 were expressed either in oral or vaginal infections, the *SAP*1-3 were preferentially expressed in vaginal, rather than oral, infections. Since then to more recent study [37], the results indicated either the differential expression of the *SAP* in humans or correlation these genes with active disease

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Table I

Expression of secreted aspartyl proteinases in human and in animal models

Model	Infection	Secreted aspartyl proteinases	Assay	Main findings	References
Murine and guinea pig	Systemic infection	*SAP*4-6	Southern blotting, survival curves	*SAP*4, *SAP*5 and *SAP*6 expression was observed during progression of systemic infection by *C. albicans* in animals	[43]
Murine and guinea pig	Disseminated infection	*SAP*1-3	Southern blotting, hybridization autoradiography	*SAP*1, *SAP*2 and *SAP*3 were detected during *C. albicans* disseminated infections	[20]
Rat	Infected rat vagina	Sap	Immunoelectron microscopy, SDS-PAGE with antirabbit IgG or antimouse IgG-peroxidase conjugated	Sap secreted by *C. albicans* during rat vaginitis; Sap localized in the cell wall of hyphal forms during infection	[53]
Human	Sera	Sap	Western blot with monoclonal antibody (MAb)	Detection of Sap antigen in the sera of patients with invasive candidias	[7]
Human	Oral candidiasis	*SAP*1-7	RT-PCR	*SAP*1-3 transcripts were observed in patients with oral candidiasis	[34]
Human	Oropharyngeal candidiasis	Sap1-3	Immunoelectron microscopy with murine monoclonal antibody	The expression of Sap1-3 does not confirm a pathogenetic role of the Sap1-3 in host-fungal interaction	[46]
Human	Oral and cutaneous candidiasis	Sap1-3 or Sap4-6	Immunoelectron microscopy	Sap1-3 crucial for mucosal and cutaneous candidiasis	[47]
Mice	Gastrointestinal infection	*SAP*1-6	RT-PCR, IVET	Expression of *SAP*4-6 was detected in higher percentage than *SAP*1-3; individual Saps are not indispensable factors for virulence	[25]
Mice	Disseminated candidiasis	*SAP*4-6	RT-PCR; immunoelectron microscopy	Hyphal morphologies without expression of *SAP*6 are less virulent; Sap1-3 antigens were found on yeast and hyphal cells, Sap4-6 were predominantly found on hyphal cells in close contact with host cells	[15]
Human	Vaginal candidiasis	*SAP*1-10	RT-PCR	*SAP*5 and *SAP*2 transcript were expressed in *C. albicans* cells infecting human epithelia *in vivo*	[48]
Mice	Keratitis infection model	*SAP*4-6	Southern blot analysis	*SAP*6 appears to be associated with morphogenetic transformation of yeast to invasive filamentous forms, *SAP*6 contributes to corneal pathogenicity	[23]
Human	Oral candidiasis	*SAP*1-6	qRT-PCR	*SAP*5 and *SAP*9 are the most highly expressed proteinase genes *in vivo*	[37]
Mice	Disseminated infections	*SAP*1-6	qRT-PCR	Expression of *SAP*1-6 was low in murine model of haematogenously disseminated candidiasis	[11]

and anatomical location. Address to the above, the letter authors [37] found that *SAP*5 and *SAP*9 are the most highly expressed proteinase genes *in vivo*.

Using animal models by Stringaro et al. [53] and RHE by Schaller et al. [46] both groups demonstrated that Sap1 and Sap2 are favoured during experimental rat vaginitis and actively secreted by *C. albicans* hyphal forms. Other studies [15, 41, 50, 51] showed that Sap4 and Sap6 are constitutively expressed during intraperitoneal infection of mouse models, while Sap2, Sap3, and Sap5 are only occasionally detected. A more recent study by Correia et al. [11] suggested that Sap1 to Sap6 do not play a significant role in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis and that Sap1 to Sap3 are not necessary for successful *C. albicans* infection. Finally, it was emphasized that Sap4-6 proteins play role during the initial phase of organ invasion, while Sap1-3 proteins play part in the later phases of the pathogenesis process. A more recent study by Jackson et al. [23] found that morphogenic conversion of *C. albicans* is closely associated with Sap6 production during corneal infection. Moreover, Jackson et al. [23] using Sap mutants suggested that *SAP*6 is involved in the pathogenesis of *C. albicans* keratitis as *SAP*6-altered strains did not establish persistent infection and failed either to invade or to trigger inflammation. Furthermore, reintroduction of the *SAP*6 gene into the fungal genome reconstituted corneal pathogenicity [23].

However, virulence differences in human data and previous mouse data (mentioned above) depend on dif-

ferent isolates of *C. albicans* and different infection models or molecular techniques employed. Animal models are powerful tools to study the pathogenesis of diverse types of candidiasis [9]. Moreover, murine models are particularly attractive because of cost, easy of handling, and experience with their use [9]. Despite of the above, there are differences in the interaction of *C. albicans* with these two mammalian species because of *C. albicans* is a natural commensal/pathogen of human but not of mice [37].

In general, Sap expression is regulated by nutritional supplementation and environmental factors, i.e., temperature, pH and the growth phase of *C. albicans* cells [35]. Chen et al. [8] determined that change of pH and temperature in the culture medium affect Sap4-6 expression. According to some authors [1, 22, 28, 46], Sap2 and Sap3 expression were optimal at pH 3.5 while Sap6 was most efficiently expressed in a less acidic (pH 5.0) medium as determined by tests conducted at 37°C in media with nitrogen source. Furthermore, according to Wu et al. [57] low levels of extracellular Sap may also stimulate the transcription and translation of *SAP* genes. Human serum provides proteins such as albumins and globulins generally considered to be cleaved by Sap enzymes which provide the essential nitrogen for cell growth [51, 55]. Early work [6] demonstrated that certain culture conditions influence proteolytic activity of *Candida* strains. The authors [6] claimed that among substrates hydrolyzed by Sap are: salivary lactoferrin, lactoperoxidase, mucin, secretory immunoglobulins, including secretory IgA. Moreover, three reports [6, 35, 46] noted that Sap2 exhibits broad substrate specificity. Their findings showed that collagen, stratum corneum, laminin and fibronectin are efficiently degraded by Sap2. The first experimental evidence [9, 10] suggests that Sap2 may be involved in the progression extracellular digestion of the intestinal mucus barrier observed after oral-intragastric inoculation of *C. albicans* in the infect mouse model. In addition, Lermann and Morschhäuser [29] found that only *SAP*2 is required for *C. albicans* growth in YCB-BSA medium (yeast carbon base – bovine serum albumin). The authors examined *sap4Δ sap5Δ sap6Δ* triple mutant and demonstrated that *SAP*2 expression does not depend on any of those other proteinases. In summary, the studies [1, 6, 20, 21, 35, 38, 46] noted that Sap2 is the most abundant secreted protein *in vitro* under growth in presence of exogenous protein. Thus, in these conditions high levels of aspartic proteases must be secreted to support growth [20].

Very little is known about *SAP*7 and *SAP*8 role in *C. albicans* pathogenesis, however according to Hornbach et al. [19] the expressions of Sap7 and Sap8 do not correlate with virulence. Furthermore, Albrecht et al. [4] note that mutants lacking *SAP*9 and/ or *SAP*10 had altered adhesion properties and were mitigated in inducing tissue damage. According to Hornbach et al. [19] expression of *SAP*9 is possibly independent of pH and morphotype. Studies of *C. albicans* gene expression during experimental infection reveal that different stress responses are mounted during different types of infections, presumably because different environments present different challenges [27]. In the future the search for Saps involvement in virulence and the molecules that determine the differences in Sap expression by quantification of experimental data, with using real-time PCR ought to be conducted.

2. Other non-*Candida albicans* species that produce aspartic proteinase

Many pathogenic non-*albicans* fungi of the genus *Candida* including such species as *Candida glabrata*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis* possess *SAP* genes [24, 36]. For example, experiments held with using *SAP1*DNA as a probe allowed identifying cross-reacting bands in the genomic DNA of *C. tropicalis*, *C. parapsilosis* and *C. guillermondii* [18, 32].

The yapsins of *S. cerevisiae* are a family of five glycosylphosphatidylinositol (GPI)-linked aspartyl proteinases (Yps1-3, Yps6, and Yps7) that have been shown to cleave peptides C-terminal to basic residues both *in vitro* and *in vivo* [24, 26]. The *S. cerevisiae YPS* genes are induced during cell wall remodelling; furthermore they have homologues in other fungi such as *C. albicans* as well as *C. glabrata* [26]. In *C. albicans*, the Yps-related proteinases are Sap9 and Sap10 [24]. Kaur et al. [24] using mouse macrophage-like cell line J774A.1 showed that macrophage-internalized *C. glabrata* exhibit transcriptional induction of a specific for this species cluster of genes encoding a family of putative aspartyl proteinases. Furthermore, 11 GPI-linked aspartyl proteinases are encoded by *C. glabrata* are closely related to *YPS* genes of *S. cerevisiae* [24]. Kaur et al. [24] noted that these genes are also required for survival within macrophages and for virulence in a murine model of disseminated candidiasis caused by *C. glabrata*. However, no typical *SAP* genes were described in *C. glabrata* [39].

Previously, the existence of the *SAPT* gene family in *C. tropicalis* was suggested [32]. Furthermore the study of Zaugg et al. [58] demonstrated that this gene family is likely to contain only four members (*SAPT1-4).* During *in vitro* studies it was observed that *C. tropicalis* show Sap activity in a medium containing bovine serum albumin as the sole source of nitrogen [57]. These authors [57] suggested that Sapt2, Sapt3, and Sapt4 could be produced under conditions yet to be described *in vitro* or during infection. However, it appears that Sapt1 plays a little role in *C. tropicalis* pathogenesis [18] as studies of

Togni et al. [55] showed that Sapt1 did not contribute significantly to fungal virulence in inoculation candidiasis of the normal mouse. Although mortality rate was slightly lower in groups infected with the *Δsapt1* strain, the mutant still possessed the ability to invade and grow in the kidneys, which eventually led to the death of the infected mice [54]. Effect of disturbing Sapt2p, Sapt3p, and Sapt4p has yet to be studied [18]. Based on phylogenetic analyses, Parra-Ortega et al. [39] demonstrated that *C. tropicalis SAPT4* is orthologous to *C. albicans SAP1* and *C. dubliniensis SAPD1.*

It has been described that *C. dublinensis* is species that is most closely related to *C. albicans* [33]. Furthermore, *C. dublinensis* strains show highly proteolytic activity by producing greater amounts of proteinase than reference *C. albicans* strains [17]. Existence of *SAP* family in *C. dublinensis* was confirmed by the study of Gilfillan et al. [17]. The latter authors [17] demonstrated that all the *C. dubliniensis* isolates examined possessed homologues to each of the seven *C. albicans SAP* genes tested. In total, eight *SAP* genes (Sapcd1-Sapcd4; Sapcd7-Sapcd10) in *C. dublinensis* were described [39]. Furthermore, *SAPD4* is only member of the Sap4-6 family found in the *C. dubliniensis* genome and the other non-*albicans Candida* species have no Sap4-6 orthologues [39]. However role of Sap isoenzymes in *C. dublinensis* pathogenesis requires further studies [18].

Candida parapsilosis exhibit proteinase activity in medium where BSA is the sole nitrogen source [13]. In the study of De Viragh et al. [13] two genes encoding putative secreted aspartic proteases were identified, though only one product was identified as extracellular aspartic proteinase [40]. Furthermore, the latter authors [13] reported the purification of *C. parapsilosis* acid proteinases produced by yeasts in BSA. In further studies of *C. parapsilosis SAP* genes, three Sap isoenzymes (Sapp1-Sapp3) had been identified [39]. However, the effect of disturbing *C. parapsilosis SAP* genes has not been investigated [18].

Although other species posses *SAP* genes, *C. albicans* aspartyl proteinase is by far most characterised in detail [36] as this yeast remains the key fungal pathogen of humans. According to Parra-Ortega et al. [39] non-pathogenic yeast species contain fewer *SAP* and *YAP* genes than their opportunistic pathogen relatives. However same authors mention that *SAP* genes expression studies of non-*albicans Candida* species such as *C. tropicalis* and *C. parapsilosis* are still missing [39].

3. Aspartic proteinase inhibitors

The role of *Candida* proteinases in pathogenesis and their potential as antifungal targets – have driven the use of aspartyl proteinase inhibitors (PIs) in *Candida* research. The studies using the classical aspartyl PI pepstatin A and HIV PIs provided evidence for the contribution of Sap to *C. albicans* virulence. Schaller et al. [44] showed that pepstatin A was able to influence adhesion and invasion of *C. albicans in vitro*. Their findings indicated that pepstatin A can reduce histological damage during *C. albicans* infection in the model of human oral candidiasis. Furthermore, this would indicate that Sap activity contributes to the virulence in this *in vitro* model. Unfortunately, it was shown [42] that classical ligand pepstatin A cannot be used clinically, at least not systematically, because of its metabolism in the liver and rapid clearance from blood. Moreover, the results obtained by Lermann and Morschhäuser [29] cannot unequivocally include the possibility that pepstatin A inhibit Sap activity under the conditions used in the RHE model. Similar results were obtained by Schild et al. [50], who demonstrated that Sap9/Sap10 exhibiting a limited inhibition potential by pepstatin A. *Candida albicans* aspartic proteinase belongs to the same family as abundant HIV proteinase, the effect of three HIV proteinase inhibitors (ritonavir, indinavir and saquinavir) was studied on *Candida* adhesion to epithelial cells [14]. Among them ritonavir was found to be the most potent inhibitor of fungal adhesion [5, 10]. The latter authors [5] concluded that although the HIV protease inhibitors were found to attenuate adhesion of *C. albicans* to epithelial cells *in vitro,* but were not able to modulate phagocytosis of cells by PLMNs. Indeed, the drug capable of blocking adhesion could prove to be attractive anti-fungal. However, later it was reviewed [14] that future derivatives designed to treat mucosal candidiasis in humans may require improvements. For more information on remain HIV PIs efficacy at inhibition of *C. albicans* proteinase activity, the reader is guided to reference by Mardegan et al. [30] and Fear et al. [14]. Another promising approach was the development of antibodies against Sap. In 2007 De Bernardis et al. [12] showed that human domain antibodies against Sap2 inhibit the adherence of *C. albicans* to epithelial cells of rat vagina and that they exert a protective activity against experimental vaginal candidiasis.

As reviewed Gauwerky et al. [16], today the obvious need for completely new antimycotic agents is clear. Thus, drug combination that target not only essential genes but also important virulence factors that are essential for steps in infection could be attractive in the treatment of candidasis.

4. Summary

The role of Saps in the development and progression of candidiasis has been studied *in vivo* for systemic and mucosal candidal infections. In addition, the research

involving both human samples and animal models were discussed (Table I). Those studies indicate that there are differences in the interaction of *C. albicans* with *in vivo* human and animal model. Moreover, it has been demonstrated that Sap mutants and specific aspartic proteinases inhibitors (i.e. pepstatin A) reduce the ability of *C. albicans* to damage host tissues [3, 6, 9, 22, 36, 55].

Although other non-*albicans Candida* species posses *SAP* genes, *C. albicans* aspartyl proteinases by far remain best characterised [36].

The knowledge on how the pathogen regulates the production of different virulence factors contributes to our better understanding of the pathogenesis [2]. Therefore further studies on *SAP* production and expression are required, which will not only help to develop more effective treatment of candidiasis but could also offer therapeutic options in the treatment of other inflammatory conditions.

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