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A novel *Candida glabrata* cell wall associated serine protease

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ABSTRACT

We set out to identify the *Candida glabrata* cell wall attached proteases which may play a role as virulence factors in candidosis, particularly in the immunocompromized host. We studied a clinical *C. glabrata* strain T-1639, which was isolated from a patient from the Helsinki University Central Hospital. With non-reducing 2-D electrophoresis using parallel fluorogenic gels and mass spectrometry we identified a novel appr. 25 kDa (192 aa in length) cell wall located protease with an estimated pI of 7.6. The LC-MS/MS peptides matched with the ORF of predicted *C. glabrata* CBS138 cell wall protein Cwp1.2p/pI 7.7/212 aa (http://cbi.labri.fr/Génolevures/[NCBI access 49525604, UniProt access Q6FTZ7]), which is an ortholog to *Saccharomyces cerevisiae* cell wall protein Cwp1p (UniProt access P28319). The novel serine protease was released by β-1,3-glucanase treatment from the cell wall. In contrast to previous predictions this protease has an enzymatic function instead of being merely a structural cell wall protein. The protease showed gelatinolytic activity and was inhibited by PMSF, a known serine protease inhibitor. Further characterization of the protease may give insight to its role in infections caused by *C. glabrata* and possibly aid in the development of new kinds of antifungal drugs.

1. Introduction

Candida glabrata (*C. glabrata*) is considered a commensal mucosal yeast, but opportunistic *C. glabrata* infections are becoming more prevalent [1]. It is the most commonly found non-*Candida albicans* species in humans and it frequently causes serious infections in immunocompromized hospital patients [2]. It is considered innately resistant to the most common antifungal group, the azoles [3]. The yeast cell wall mannoprotein architecture influences antifungal susceptibility [4]. Considering this, detailed structural information of virulence factors give clues to the development of fungal inhibitors [5].

C. glabrata is phylogenetically closer related to *Saccharomyces cerevisiae* (*S. cerevisiae*) than to *Candida albicans* (*C. albicans*) [6] and has a unique set of virulence mechanisms [7], such as phenotypic switching [8], and pseudohyphal formation [9], no secreted proteases but phospholipase activity [10], and adhesins [11]. The role of *Candida* proteases in virulence is still unclear, but there is evidence that strain-dependent variations exist among yeasts [12,13]. The function of one of the GPI-linked aspartyl proteases, CgYps1, has been recently shown to participate in the regulation of pH homeostasis in *C. glabrata* [14]. The glycosylphosphatidylinositol-linked aspartyl proteases of *C. glabrata* have a role in yeast cell wall remodeling and adhesion properties [15].

Basically *C. glabrata* cell wall resembles *C. albicans* and *S. cerevisiae* bilayered cell wall structure [16,17,18,19]. The outermost cell wall layer structure of *C. glabrata* is less well characterized and it contains mainly mannoproteins and the cross-linking of the proteins is different compared to *C. albicans* and *S. cerevisiae* [20]. *C. glabrata* cell wall contains 50% more protein than *S. cerevisiae* cell wall and have higher mannose/glucose ratio. *S. cerevisiae* expresses yapsins which participate in the remodeling of the yeast cell wall through glucan homeostasis [21].

The cell wall contains covalently linked glycosylphosphatidylinositol (GPI)-proteins and mild-alkali-sensitive cell wall proteins (CWPs) which are attached to a 1,6-β-glucan/1,3-β-glucan network of the cell wall [22]. This *in silico* study of *C. glabrata* cell wall proteins predicted 106 putative GPI-proteins categorized as adhesive proteins, hydrolases, phospholipases, aspartic proteases and structural cell wall proteins. In the study GPI-CWPs were also extracted using HF-pyridine (which

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specifically cleaves phosphodiester bonds) and further immunological analysis of this extract confirmed the presence of two predicted *C. glabrata* cell wall proteins: Cwp1.1p and Cwp1.2p.

To this background we focused in the present study on clinical blood isolates of *C. glabrata* and on the proteases which might locate on their outermost cell wall layer.

2. Materials and methods

2.1. Preparation of the cell wall extract

As *Candida* species we used *C. glabrata* blood isolates from the Helsinki University Central Hospital. The yeast cells were grown 24 h on SAB- dextrose-agar (Lab 4, Bury, UK). Colonies from this plate were further grown in YPG (0.5% yeast extract, 1% peptone and 0.5% glucose) in a water bath for 24 h at 37 °C. The cells were then washed twice with 5 ml PBS (phosphate buffered saline), centrifuged (200 g, 10 min, RT), and the cell density was adjusted to 10^7 cells/ml. The cells were in non-pseudohyphal form confirmed by phase-contrast microscopy.

The extraction of cell wall proteins was performed as described by Klinke et al. [23] with slight modifications. The cells were incubated in 30 U/ml lyticase (*Arthrobacter luteus*, Sigma–Aldrich, St. Louis, MO) at 31 °C for 24 h on a shaker, centrifuged (200 g, 10 min, RT) and the supernatant (cell wall extract) was stored at −80 °C. The same procedure was repeated (22.5 h) and the supernatants were pooled and filtered (0.22 μm, Millipore, Billerica, MA).

Gelatinolytic activity was estimated by MDPF (2-methoxy-2,4-dephenyl-3 (2H)furanone) –gelatin (1%, v/v)-zymography (8% SDS-PAGE gel) as in Pärnänen et al. [24]. Briefly, cell wall extracts were run by nonreducive MDPF-gelatin zymography with or without 1 mM, 5 mM and 10 mM serine protease inhibitor PMSF (phenylemethanesulphonylfluoride, Sigma–Aldrich, St. Louis, MO) and 5 mM EDTA. The ability of the cell wall extract to degrade L-Arg-AMC (7-amino-4-methylcoumarin; Sigma–Aldrich, St. Louis, MO) was also tested as described by Rodier et al. [25]. Briefly, 10 μl of non-concentrated cell wall extract was incubated with 8 μl of L-Arg-AMC (final concentration 20 mM) as according to manufacturer's instructions. The most gelatinolytic strain T-1639 was used in further studies.

2.2. 2-D electrophoresis and zymography

The first dimension isoelectric focusing was performed with an Immobiline™ Dry Strip (pH 3–10, 13 cm; GE Healthcare, Uppsala, Sweden). The second dimension SDS-PAGE (11%) was run in non-reducive conditions without DTT to maintain potential protease activity at RT for 26 h. A parallel SDS-PAGE (11%) gel with 1% (v/v) MDPF-gelatin as a substrate was also run. The gel for mass spectrometric analysis was silver stained immediately after the run. The parallel gel was incubated in zymobuffers as in Pärnänen et al. [24]. After 4 days the gelatinolytic activity was matched with the corresponding sites in the silver stained gel and areas indicating gelatinolytic activity were cut out.

2.3. Mass analysis

Samples cystein bonds were reduced with 0.05 M dithiothreitol (#D0632 Sigma–Aldrich, USA) for 20 min at 37 °C and alkylated with 0.15 M iodoacetamide (#57670 Fluka, Sigma–Aldrich, USA) at room temperature. Samples were digested by adding 0.75 μg trypsin (Sequencing Grade Modified Trypsin, V5111, Promega). After digestion the peptides were purified with C18 microspin columns (Harvard Apparatus) according to the manufacture's protocol and re-dissolved in 30 μl.

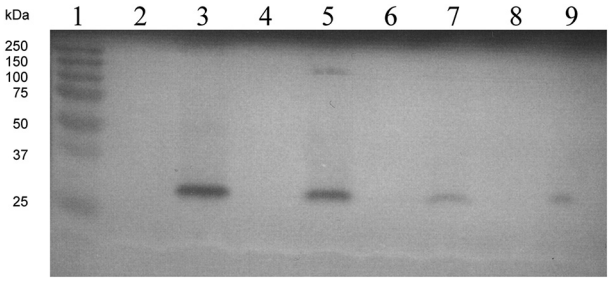

Fig. 1. Cell wall appr. 25 kDa protease inhibition with PMSF. Lane 1. Molecular weight standard (kDa). Lanes 2, 4, 6 and 8 empty. Lane 3. Cell wall lyticase (CWL) fraction without PMSF. Lane 5. CWL + 1 mM PMSF. Lane 7. CWL + 5 mM PMSF. Lane 9. CWL + 10 mM PMSF.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was carried out on an EASY-nLC (Thermo Fisher Scientific, Germany) connected to an Orbitrap Elite spectrometer (Thermo Fisher Scientific, Germany) with nano-electrospray ion source (Thermo Fisher Scientific, Germany). For the protein identification the acquired MS2 scans were searched against the corresponding protein sequence entries from the UniProt protein database using the Sequest search algorithms via the Thermo Proteome Discoverer software (Thermo Fisher Scientific, Germany). Allowed mass error for the precursor ions was 15 ppm, and for the fragment ion 0.8 Da.

2.4. Secondary structure prediction

C. glabrata Q6FTZ7 secondary structure prediction was made with http://bioinf.cs.ucl.ac.uk/psipred and comparison of *C. glabrata* Cwp 1.2p with *S. cerevisiae* Cwp1p was made with CFSSP (Chou & Fassman Secondary Structure Prediction Server, www.expasy.org). The prediction of the protein mass and pI were estimated with www.expasy.org Compute pI/Mw-tool, signal peptide with www.cbs.dtu.dk SignalP 4.1. Server, hydrophobicity with www.expasy.org/protparam tool, transmembrane helixes and PIR-proteins (proteins with internal repeats indicating a GPI- anchor) with the proFASTA web tool (http://www.bioinformatics.nl/tools/profasta) [26].

3. Results

In order to evaluate enzymatic activity the following results were obtained. Lyticase-treatment caused liberated gelatinolytic activity of the cell wall extract is shown in Fig. 1. Gelatinolysis appeared at appr. 25 kDa area and inhibition of this activity was noticed with the serine protease inhibitor PMSF (5–10 mM). EDTA did not affect the proteolytic activity (results not shown). The L-Arg-AMC- degradation assay was also used to verify overall activity

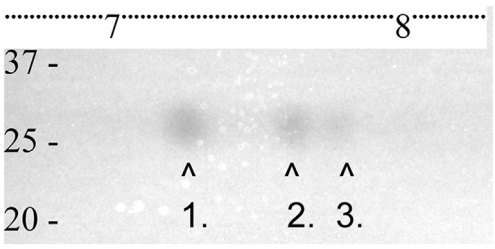

Fig. 2. MDPF-gelatin 2D-SDS-PAGE of the cell wall lyticase-fraction. 1., 2., and 3. gelatinolytic areas are marked with arrowheads. Isoelectric focusing strip pH range is indicated in the upper border. Molecular weight marker (kDa) located on the vertical left.

Table 1
LC-MS/MS data search results from NCBI- and UniProt −protein databases.

	Description	Score	Coverage	# Proteins	# Unique peptides	# peptides	# PSMs	# AAs	MW (kDa)	Calc. pI
NCBI accession 49525604	Unnamed protein product [*Candida glabrata*]	24.01	31.13	2	4	4	7	212	20.7	7.40
UniProt accession Q6FTZ7	Strain CBS138 chromosome F complete sequence OS = *C. glabrata* (strain ATCC 2001/ CBS 138/JCM 3761/NBRC 0622/NRRL Y-65) GN = CAGL0F07579 g PE = 4 SV = 1 − [Q6FTZ7_CANGA]	24.01	31.13	2	4	4	7	212	20.7	7.40

of the cell wall extract (results not shown). The MDPF-gelatinolysis in the 2-D gel is shown in Fig. 2. There were three separate proteins of approximate size 25 kDa and a pI of approximate 7.2–7.8.

A closer look of the 2-D gel disclosed that the protease has seemingly mannoprotein extensions of different sizes which make the proteins to locate horizontally in a row as glycoforms with slight differences in pI. The middle gelatinolytic area gave the best coverage of the predicted sequence in MS/MS analysis (Table 1). The predicted molecular weight was 20.7 kDa and pI 7.4. The theoretical pI is 6.09 (without the signal sequence), but probable mannan/oligosaccharides increase pI and molecular weight compared to the observed values seen in zymography. The BLAST sequence alignment data of *C. glabrata* Cwp1.2p (Q6FTZ7, 212 aa) with *S. cerevisiae* Cwp1p (239 aa) is shown in Fig. 3. The peptides resulting from our LC-MS/MS are indicated above the aforementioned ORFs in blue and the coverage was 31.1% compared to *C. glabrata* Cwp1.2p. When comparing the overall amino acid composition of the predicted *C. glabrata* Cwp1.2p to Uniprot/P28319 *S. cerevisiae* YKL09w CWP1 cell wall mannoprotein (http://cbi.labri.fr/Génolevures/) there was 92% query coverage and identity 49%.

Finally, secondary structure and localization predictions were executed. Analyzing of this novel protease sequence with ProFASTA web tool predicted that the protein has no transmembrane domains and no glutamine or serine/threonine rich sequence indicating that it might not be a GPI-protein. There is one signal peptide cleavage sight between positions 20 and 21 from the N-terminal end (Fig. 4A), and a hydrophobic tail (Fig. 4B). Similarity in the structure is seen in *S. cerevisiae* Cwp1p. The secondary structure is predicted to have alpha-helical structures in the N-terminal signal sequence (aa 1–20) and C-terminal end. The rest of the protein is composed of alternating coils and strands and compared to *S. cerevisiae* Cwp1 the overall structure is quite similar (not shown). The existence of a signal sequence in the ORF backs up the cell wall localization of the protease. NCBI and UniProt database search results from our LC-MS/MS are given in Table 1. The molecular weight (20.7 kDa) and pI (7.4) predictions are similar in both and are in concordance with our results.

4. Discussion

The novel protease is the *in silico* predicted Cwp 1.2p of *C. glabrata* which is an ortholog to *S. cerevisiae* Cwp1p. The observed molecular weight from the gel separations of the protease is 25 kDa, whereas the predicted size of Cwp1.2p is 20.7 kDa.The signal sequence weight (amino acids 1-20) has to be subtracted from the predicted value and the obvious extra weight of mannans/oligosaccharides attached to the protein has to be added to the weight of this predicted protein. This explains the difference between the observed and predicted molecular weights. The novel protease is attached by a β-1,3-glycosidic bond to the *C. glabrata* cell

Fig. 3. ORF comparison with NCBI/BLAST. *C. glabrata* unknown protein Cwp1.2p (212aa), (NCBI access 49525604; UniProt access Q6FTZ7) in chromosome F, 1–212; Genolevures ORF numbers CAGL0F07579g/CAGLIPF543 (http://cbi.labri.fr/Génolevures) alignment with *S. cerevisiae* CWP1p cell wall mannoprotein (UniProt P28319 YKL096w CWP1 cell wall mannoprotein). The peptides obtained from our LC-MS/MS are indicated above the *C. glabrata* Cwp1.2p in blue (aa coverage 31, 1%).

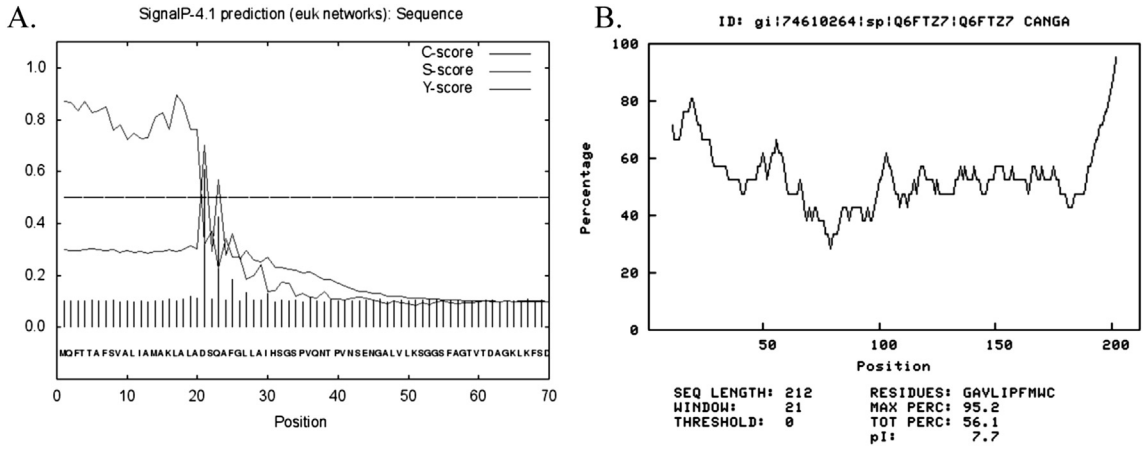

Fig. 4. Signal peptide prediction showing one signal peptide cleavage site between aa:s 20 and 21 (ALA-DS) (www.cbs.dtu.dk SignalP 4.1 Server) of *C. glabrata* Cwp1.2p (A.). Hydrophobicity prediction showing hydrophobic tail in *C. glabrata* Cwp1.2p (B.) (http://www.bioinformatics.nl/tools/profasta).

wall and the enzymatic method (lyticase treatment) we used in our study releases the protease in active form. The presence of a signal sequence and the hydrophobic tail also back up this localization. To our knowledge this localization has not been earlier demonstrated and gives thus insight into the cell wall architecture of *C. glabrata*.

The localization of cell wall proteins has an impact on host innate immune system pathogen recognition as described for *C. albicans* [27,28]. *C. glabrata* is not dimorphous (no true hyphal form) compared to *C. albicans*, which presents in both yeast and hyphal forms in certain stages of the infection process. The distinct proteome of the different yeast species is an evolutionary adaptation of the pathogens to utilize nutrients and survive in the host. This may be expressed in such regimens as virulence factor proteases. Comparing the overall secondary structure predictions between *C. glabrata* Cwp1.2p and *S. cerevisiae* Cwp1p give quite similar results, but certain differences may affect protein function. However, despite the sequence similarity of *C. glabrata* with *S. cerevisiae*, there must be crucial differences in the architecture of the cell wall layer participating in virulence because *S. cerevisiae* rarely causes serious infections. The glycosylation variations of the cell wall are complex and are of interest and further characterization of them might give new knowledge to the interaction and cell surface reaction mechanisms of the host cell to *C. glabrata* contact in infection.

The non-reducing 2-D electrophoresis method we used in this study (compared to conventional 2-D electrophoresis) maintains the searched enzyme in active form, aids in localizing protease activity areas from the gel and further identification by mass analysis. This method can be used also for scanning protease activity in other microbial species. The characterization of the novel serine protease compared to reference strain derived similar proteases might help to find possible inhibitors and eventually lead to new kinds of drugs against *C. glabrata* infections.

Conflict of interest

The authors report no conflicts of interest.

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The LC-MS/MS analyses were made at the Institute of Biotechnology, University of Helsinki.

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