

Isolation, characterization and regulation of moonlighting proteases from *Candida glabrata* cell wall

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ABSTRACT

Candida glabrata (*C. glabrata*) cell wall proteins play a role in virulence and in initial host immune recognition and responses. We isolated and characterized *C. glabrata* cell wall proteases from a clinical hospital *C. glabrata* T-1638 blood isolate and estimated the enzymatic activities and their ability to degrade gelatin and processing proMMP-8 and assess the regulation of these proteases with salt treatment, mercaptoethanol and fermented lingonberry juice from *Vaccinium vitis idaea* L. The cell wall proteases were enzymatically released from the cell wall and beta-1,3-bonded proteases were fractionated into 10–50 kDa and >50 kDa fractions with anionic DEAE-sepharose ion-exchange chromatography and gel filtration. Proteins were monitored and analyzed with MDPF-zymography, and five gelatinolytic bands were cut out from a parallel silver-stained gel for the LC-MS/MS analysis. The proteases lacked a signal sequence, indicating that they are moonlighting proteases. Human proMMP-8 activation assays were performed with both fractions and verified by western-immunoblot using aMMP-8 specific antibody. Inhibition of proMMP-8 conversion to the lower molecular active enzyme species were demonstrated with fermented lingonberry juice. The results indicate that moonlighting proteases may play a role in the virulence of *C. glabrata*.

1. Introduction

Traditionally *C. glabrata* cell wall proteins are classified into GPI- and PIR-proteins [1,2]. *C. glabrata* has not been shown to secrete proteases, such as SAPs (secreted aspartic proteases) found from *C. albicans*, but certain studies show moonlighting proteins in the cell wall of *C. albicans* [3] and *C. glabrata* [4,5]. These intracellular proteins are secreted by a non-classical pathway in extracellular vesicles (ECVs) and are found in several microbial species attached to the cell wall [6–8]. The concept of intracellular proteins having an extracellular function has given an insight into protein molecules containing multiple functions compared to what is traditionally shown [9]. Studies with *Saccharomyces cerevisiae* also show cell wall proteins found both in the cytosol and fungal cell wall [10]. *C. glabrata* cell wall proteins are in the first contact line with the host and eventually can contribute to the virulence of the organism and host immune responses.

Proteases of microbial species e.g. periodontopathogenic bacteria *Porphyromonas gingivalis* and *Treponema denticola* have been shown the

ability to degrade collagen fragments and activate latent host proMMP-1 and pro-MMP-8 [11], and *Treponema denticola* chymotrypsin-like proteinase has the ability to convert proMMP-8 and -9 into their active forms [12]. *Pseudomonas aeruginosa* elastase, *Vibrio cholera* proteinase and thermolysin can activate proMMP-1, proMMP-8 and proMMP-9 [13], and *Tannerella forsythia* chymotrypsin-like serine protease membrane bound enzyme degrades gelatin and type-I collagen [14]. Additionally, a *Candida albicans* cytoplasmic metalloprotease could completely degrade type I collagen and fibronectin, and partially degrade type IV collagen and laminin [15]. Several candidal species have been shown to possess cell wall associated/proteases secreted into the culture medium able to degrade or modify human derived basement membrane and extracellular proteins: laminin-332, fibronectin, E-cadherin, tissue inhibitor of metalloprotease-1 (TIMP-1) and proMMP-9 [16–20].

To this background we isolated *C. glabrata* cell wall proteases to determine and characterize their activities.

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2. Results

Fig. 1 Shows the effect of salt and 1.5 mM mercaptoethanol (ME)-treatments on the >50 kDa candidal fractions.

The effects of ME treatment of the fractions are shown in Fig. 2.

The bands showing gelatinolytic activity were cut out of the silver stained gel (Fig. 3a and 3b.). Distinct gelatinolytic activity bands were detected within 15.7 kDa–85.9 kDa range and these were identified using the Uniprot *C. glabrata* protein database. We identified five proteases from the >50 kDa fraction without a traditional signal peptide: glucose- 6- phosphate isomerase (GPI) Q6FRW1 (61.3 kDa, coverage 41.98%, score 41.71) (Fig. 3b band 4); glutamate dehydrogenase (GDH) Q6FWH3 (49.7 kDa, coverage 31.96%, score 65.03) (Fig. 3a band 2 and Fig. 3b band 3); 5-methyltetrahydropteroyltriglutamate - homocysteine methyltransferase (Met6), Q6FQM7 (85.9 kDa, coverage 31.29%, score 24.97) (Fig. 3a band 1); transaldolase Q6FXG5 (36.7 kDa, coverage 30.84%, score 46.95) (Fig. 3b band 5); phosphoglycerate kinase (PGK) Q6FKY1 (44.6 kDa, coverage 42.79%, score 6.61) (Fig. 3a band 3).

Both the 10–50 kDa and especially the >50 kDa proteases fragmented the 70–75 kDa latent full-size human proMMP-8 into its 30, 40, 55 kDa lower molecular weight active species (Fig. 4a.). This proMMP-8 fragmentation by candidal proteases could be inhibited by the fermented lingonberry juice in the >50 kDa isolated candidal enzyme fraction (Fig. 4b.). The 10–50 kDa fraction data is not shown.

3. Discussion

Our results indicate, that *C. glabrata* possesses moonlighting proteases in the cell wall. Several moonlighting proteases have been identified from *C. glabrata* [21]. These findings corroborate with our results. Some findings indicate cell wall proteins of *C. albicans* having properties affecting virulence in challenged environments, such as starvation and temperature upshift [22]. This may be the case also in *C. glabrata* virulence. Glucose utilization is crucial for microbial growth and survival, e. g. the proper function of GDH (glutamate dehydrogenase) in *Staphylococcus aureus* gives it enhanced ability to use glutamate as an energy source in abscesses etc. with limited glucose availability [23].

The inactivation of the >50 kDa proteases with mercaptoethanol showed, that there are intra/intermolecular disulphide bridges crucial for the gelatinolytic activity. The salt treatment of the fraction indicates some ionic bond-related activation of the gelatinolysis.

Inactivation of the 10–50 kDa and >50 kDa fractions enzymatic

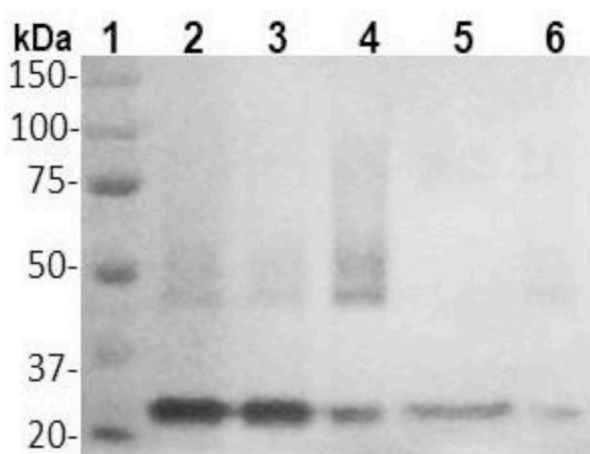


Fig. 1. SDS-PAGE of *C. glabrata* protease fractions with and without salt/mercaptoethanol (ME) exposure. Lane 1. MW standards, 2. fraction before DEAE- Sephadex, 3. fraction before DEAE- Sephadex, ME-treated (1.5 mM), 4. fraction before DEAE- Sephadex, salt treated, 5. fraction before DEAE- Sephadex, salt and ME (1.5 mM) treated, 6.10–50 kDa fraction before DEAE.

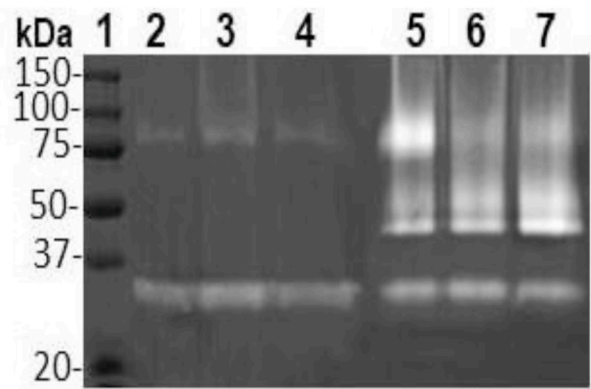


Fig. 2. Gelatin-zymography of DEAE- Sephadex/G-200 fractions of *Candida glabrata* protease purification with and without mercaptoethanol (ME). Lane 1. Mw standards, 2. Fraction 8 (DEAE+ 1,5 mM ME), 3. Fr 3 (DEAE + G-200 + 1,5 mM ME), 4. Fr 4 (DEAE + G-200 + 1,5 mM ME), 5. Fr 8 (DEAE, without ME), 6. Fr 3 (DEAE + G-200, without ME), 7. Fr 4 (DEAE + G-200, without ME).

activity with fermented lingonberry juice presents a potential therapeutic agent against *C. glabrata* virulence. Natural substances are being increasingly tested for antimicrobial properties. An interesting study has shown that mammalian glutamate dehydrogenase could be inhibited by green tea phenolics [24]. Fermented lingonberry juice also contains polyphenols, and further studies are needed to establish the inhibitory factor(s) from lingonberry. Recently, a clinical pilot study revealed the reduction of oral fluid aMMP-8 in the oral cavity by a lingonberry oral mouthwash intervention and management [25].

The gelatinolytic activity and activation of human proMMP-8 by *C. glabrata* cell wall associated moonlighting proteases may be directly involved in the local connective tissue breakdown as well as induction and modulation of the inflammatory immune responses in the affected tissues by the invading yeast. Inhibition of this activity by fermented lingonberry juice *in vitro* may have clinical implications in *C. glabrata* infection control. The anti-inflammatory properties of fermented lingonberry juice are to be further investigated.

4. Materials and methods

4.1. Preparation of the *C. glabrata* cell wall fractions

C. glabrata T-1638 was a blood isolate from a Helsinki University Hospital patient. By preliminary gelatin-zymographic scanning of several blood isolates T-1638 showed highest gelatinolytic enzymatic activity. The cell wall extract was prepared by the following methods. *Candida* cells were first grown on Sabouraud dextrose agar (Lab M, Bury, UK) 24 h at 37 °C, then in 4 l yeast (0.5%) peptone (1%) glucose (1%) (YPG) 24 h at 37 °C on a waterbath with shaking. After filtering (Millipore Express™ Plus; 0,22 µm; Merck, Darmstadt, Germany) the cells were washed and centrifuged (3095 g, 10 min, RT) twice with PBS (phosphate buffered saline). The cells were incubated with 30 U/ml lyticase (Sigma-Aldrich, St Louis, MO) for 22 h at 31 °C in PBS on gentle shaking and centrifuged (3095 g, RT). The supernatant was stored at –20 °C and volume was restored with PBS. The cells were incubated a second time with lyticase similarly and the obtained supernatants were pooled (cell wall fraction). The cell wall fraction was centrifuged with 10 kDa and 50 kDa cutoff filters according to the manufacturer's instructions (Amicon® Ultra- 15, Millipore, Billerica, MA), fraction volumes were restored with PBS and proteins were recovered from the filter to obtain 10–50 kDa and >50 kDa fractions.

4.2. Gelatin-zymography

To estimate gelatinolytic activity of the fractions, 8% MDPF (2-

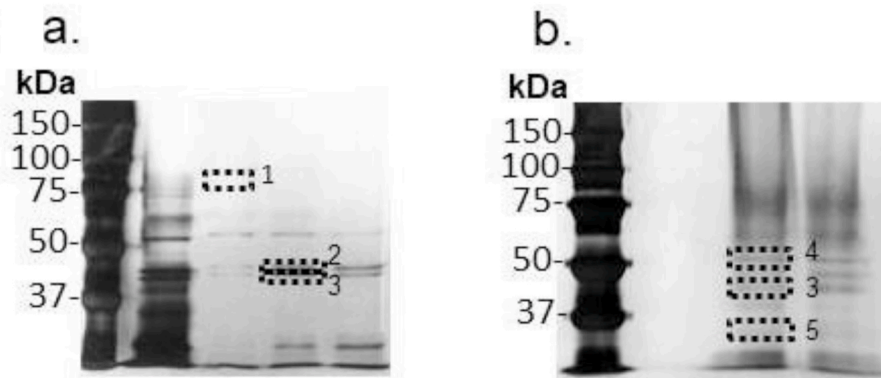


Fig. 3. Silver stained 8% SDS-PAGE analyses of fractions sequenced. Fig. 3a. 1: Met 6 (85.9 kDa); 2: GDH (49.7 kDa); 3: PGK (44.6 kDa); Fig. 3b. 4: GPI (61.3 kDa); 3: GDH (49.7 kDa); 5: transaldolase (36.7 kDa).

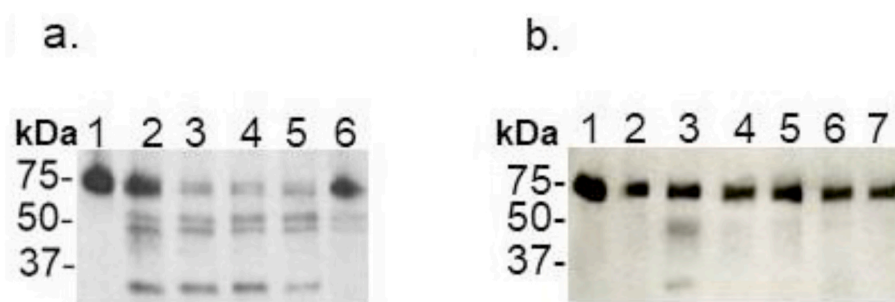


Fig. 4. Western immunoblot analysis of the effects of >50 kDa *Candida glabrata* protease fraction with and without Lingora® on purified latent 75 kDa human proMMP-8. Incubation times indicated. Fig. 4a: Lane 1. proMMP-8; 2. proMMP-8+ > 50 kDa fraction, 30 min; 3. proMMP-8+ > 50 kDa fraction, 1 h; 4. proMMP-8+ > 50 kDa fraction, 2 h; 5. proMMP-8+ > 50 kDa fraction, 5 h; 6. proMMP-8+ APMA, 1 h. Fig. 4b: Lane 1. proMMP-8; 2. proMMP-8+ 5 µl Lingora®, 30 min; 3. proMMP-8+ > 50 kDa fraction, 30 min; 4. proMMP-8+ > 50 kDa fraction + Lingora®, 30 min; 5. proMMP-8+ > 50 kDa fraction + Lingora®, 1 h; 6. proMMP-8+ > 50 kDa fraction + Lingora®, 2 h; 7. proMMP-8+ > 50 kDa fraction + Lingora®, 5 h. Mobilities of the molecular weight standards are indicated on the left.

methoxy-2,4-diphenyl-3 (2H) -furanone)- gelatin (1%) zymography was performed according to Pärnänen et al. [17]. The original cell wall extract and the >50 kDa fraction was treated also with 2 µl β-mercaptoethanol (final concentration = 1.5 mM) at 37 °C for 1 h. A parallel 8% SDS- PAGE was run.

4.3. Salt and mercaptoethanol (ME) treatments

To estimate ionic or disulfide bonds in the enzyme, 100 µl of the >50 kDa fraction was incubated in 1 ml 20 mM Tris-HCl, pH 8.2 + 0.5 M NaCl for 1 h, centrifuged with a Millipore 10–50 kDa cutoff device (3095 g, 10 min, RT) and washed twice. The flowthrough was also collected and concentrated 10 × with a Millipore 10 kDa cutoff device. Samples of the >50 kDa fraction and the salt-treated fractions were incubated with or without 2 µl of β-mercaptoethanol (final concentration = 1.5 mM) for 1 h (37 °C). Samples were run on 8% MDPF-gelatin zymography.

4.4. Ion-exchange chromatography

Tha buffer of the >50 kDa fraction was changed to 20 mM Tris-HCl, pH 8.2. Ion-exchange with diethylaminoethyl cellulose chromatography for the >50 kDa cell wall extract was performed according to the manufacturer's instructions (DEAE- FF, HiTrap® IEX Selection kit, GE Healthcare, Chicago, IL). The proteases were eluted with 0.5 M NaCl in 20 mM Tris-HCl, pH 8.2.

4.5. Gel filtration

A 15 cm column (V = 9.5 ml) was used for gel filtration. Sephadex G-100 filtration for both 10–50 kDa and >50 kDa fractions, and Sephadex

G-200 filtration for the >50 kDa fraction were performed.

Inhibition of gelatinolytic enzymatic activities were tested with 2 µl β-mercaptoethanol (final concentration = 1.5 mM) (incubated 2 h, 37 °C).

Parallel 8% SDS-PAGE with silver stain were run.

4.6. ProMMP-8 conversion and inhibition assays

10–50 kDa and >50 kDa isolated candidal protease fractions were tested for the ability to convert and fragment the full-size 70–75 kDa human proMMP-8. 8.5 µl of the fractions were incubated with 0.8 µg of 70–75 kDa proMMP-8 (R&D System, Oxon, UK) for 0, 1, 2, 5 h, o/n at 37 °C. As positive control, proMMP-8 was treated and activated by 1 mM aminophenylmercuric acetate (APMA) used as described by Nieminen et al. [12]. Inhibition of the activity was performed with the same timeframe using locally produced fermented lingonberry juice, final concentration 5 µg/µl (Lingora®; 0,1 µg/µl; Vantaa, Finland [25,26]).

4.7. Western immunoblot

The detection of proMMP-8 conversion and inhibition was verified by a modified enhanced chemiluminescence (ECL) Western blotting kit according to the protocol recommended by the manufacturer (GE Healthcare, Buckinghamshire, UK). As specific primary and secondary antibodies were performed polyclonal anti- MMP-8 [27] and anti-rabbit IgG horseradish peroxidase-linked (GE Healthcare, Buckinghamshire, UK), respectively [12,28].

4.8. Mass analysis

Parallel 8% SDS-PAGE gels were silver-stained [29], protein bands

were cut out of the polyacrylamide gel and “in-gel” digested. Cystein bonds were reduced with 0.045 M dithiothreitol (#D0632 Sigma-Aldrich, St. Louis, MO) for 20 min at 37 °C and alkylated with 0.1 M iodoacetamide (#57670 Sigma-Aldrich, St. Louis, MO) at room temperature. Samples were digested by adding 0.75 µg trypsin (Sequencing Grade Modified Trypsin, V5111, Promega) for overnight at 37 °C. After digestion peptides were purified with C18 microspin columns (Harvard Apparatus, Holliston, MA) and the dried peptides were reconstituted in 30 µl of buffer A [0.1% trifluoroacetic acid (TFA) in 1% acetonitrile].

Mass analysis was performed as in El Omar et al. [30], but with a 6 µl sample size. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis was carried out on an EASY-nLC1000 (Thermo Fisher Scientific, Darmstadt, Germany) connected to a Velos Pro-Orbitrap Elite hybrid mass spectrometer (Thermo Fisher Scientific, Germany) with nano electrospray ion source (Thermo Fisher Scientific, Germany). The LC-MS/MS samples were separated using a two-column setup consisting of a 2 cm C18-Pepmap trap column (Thermo Fisher Scientific, Germany), followed by 15 cm C18-Pepmap analytical column (Thermo Fisher Scientific, Germany). The linear separation gradient consisted of 5% buffer B in 5 min, 35% buffer B in 60 min, 80% buffer B in 5 min and 100% buffer B in 10 min at a flow rate of 0.3 µl/min (buffer A: 0.1% TFA in 1% acetonitrile; buffer B: 0.1% TFA acid in 98% acetonitrile). 6 µl of sample was injected per LC-MS/MS run and analyzed. Full MS scan was acquired with a resolution of 60 000 at normal mass range in the orbitrap analyzer and followed with CID-MS2 top 20 most intense precursor ions within ion trap (energy 35). Data was acquired using LTQ Tune software.

Acquired MS2 scans were searched against Uniprot *Candida glabrata* protein database using the Sequest search algorithms in Thermo Proteome Discoverer. Allowed mass error for the precursor ions was 15 ppm and for the fragment 0.8 Da. A static residue modification parameter was set for carbamidomethyl +57.021 Da (C) of cysteine residue. Methionine oxidation was set as dynamic modification +15.995 Da (M). Only full-tryptic peptides were allowed for maximum of 1 missed cleavages were considered.

Author contributions

The authors P.P, T.S, T.T. and P. N-I. participated in planning, analysis of the data and reviewing the manuscript. The author P.P. performed the sample preparation and other characterizations of *C. glabrata* cell wall proteases, except western immunoblots and proMMP-8 conversion and inhibition assays which were performed by the author T.T.

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Declaration of competing interest

The author P.P. is the inventor and holder of the patent EP 2 585 087 B1. The author T.S. is the inventor of US patents 5652227, 5866432, 6143476, 2017002357A1 and PI758USPC. The authors declare that there are no conflicts of interest.

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