



Original article

Vacuolar proteases from *Candida glabrata*: Acid aspartic protease PrA, neutral serine protease PrB and serine carboxypeptidase CpY. The nitrogen source influences their level of expression



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ABSTRACT

Background: The *Saccharomyces cerevisiae* vacuole is actively involved in the mechanism of autophagy and is important in homeostasis, degradation, turnover, detoxification and protection under stressful conditions. In contrast, vacuolar proteases have not been fully studied in phylogenetically related *Candida glabrata*.

Aims: The present paper is the first report on proteolytic activity in the *C. glabrata* vacuole.

Methods: Biochemical studies in *C. glabrata* have highlighted the presence of different kinds of intracellular proteolytic activity: acid aspartyl proteinase (PrA) acts on substrates such as albumin and denatured acid hemoglobin, neutral serine protease (PrB) on collagen-type hide powder azure, and serine carboxypeptidase (CpY) on N-benzoyl-tyr-pNA.

Results: Our results showed a subcellular fraction with highly specific enzymatic activity for these three proteases, which allowed to confirm its vacuolar location. Expression analyses were performed in the genes *CgPEP4* (*CgAPR1*), *CgPRB1* and *CgCPY1* (*CgPRC*), coding for vacuolar aspartic protease A, neutral protease B and carboxypeptidase Y, respectively. The results show a differential regulation of protease expression depending on the nitrogen source.

Conclusions: The proteases encoded by genes *CgPEP4*, *CgPRB1* and *CgCPY1* from *C. glabrata* could participate in the process of autophagy and survival of this opportunistic pathogen.

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Proteasas vacuolares de *Candida glabrata*: aspartil proteasa ácida PrA, proteasa neutra PrB y carboxipeptidasa CpY. La fuente de nitrógeno influye en sus niveles de expresión

RESUMEN

Antecedentes: La vacuola de *Saccharomyces cerevisiae* está involucrada activamente en el mecanismo de autofagia, desarrollando una labor importante en la homeostasis, degradación, recambio proteico, desintoxicación y protección de la célula en condiciones de estrés. Por el contrario, las proteasas vacuolares de *Candida glabrata* aún no han sido estudiadas por completo.

Objetivos: El presente trabajo describe por primera vez la actividad proteolítica vacuolar en *C. glabrata*.

Métodos: Los estudios bioquímicos realizados en *C. glabrata* pusieron de manifiesto la presencia de diferentes actividades proteolíticas: aspartil proteinasa ácida, que actúa sobre sustratos como la albúmina

Palabras clave:

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y la hemoglobina ácida desnaturalizada; serín proteasa neutra, con actividad sobre el sustrato de tipo colágeno *hide powder azure*, y serín carboxipeptidasa, que actúa sobre N-benzoil-tyr-pNa.

Resultados: La obtención de una fracción subcelular mostró una elevada actividad enzimática específica de las tres proteasas, lo que permitió confirmar su localización vacuolar. Se realizaron análisis de la expresión de los genes *CgPEP4* (*CgAPR1*), *CgPRB1* y *CgCPY1* (*CgPRC1*), codificantes de las actividades proteolíticas aspartil proteasa A, proteasa neutra B y carboxipeptidasa Y, respectivamente. Los resultados reflejan una regulación diferencial de la expresión de la proteasa, dependiendo de la fuente de nitrógeno.

Conclusiones: Las proteasas codificadas por los genes *CgPEP4*, *CgPRB1* y *CgCPY1* podrían participar en el proceso de autofagia y supervivencia de este patógeno oportunista.

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The fungal vacuole is a dynamic and acidic compartment of the cell, similar to lysosomes, with a wide variety of hydrolytic enzymes such as proteases, trehalase, alpha-mannosidase, and alkaline phosphatase. The main functions of these enzymes are the degradation of proteins, storage and bidirectional transport of ions and metabolites, detoxification, ion homeostasis, and maintenance of cytosolic pH.^{15,19,23,30,33} The *Saccharomyces cerevisiae* vacuole is an important organelle for the adaptation to new environments. Its morphology changes readily for the purpose of survival in response to extracellular signals, as well as signals related to stress conditions and cell differentiation.^{2,19,40,44} In *Candida albicans*, the vacuole allows the activation of certain virulence factors, such as differentiation of the yeast-hyphae, as well as adhesion and survival within macrophages.^{17,27,29}

Candida glabrata, an opportunistic yeast pathogen that causes candidiasis, has an unknown sexual cycle. The *C. glabrata* haploid genome is phylogenetically more related to *S. cerevisiae*, with approximately 12.3 Mb organized in 13 chromosomes.^{5,7,8,31} In order to survive, proliferate and evade host defense strategies, this yeast must be able to adapt to changes in the microenvironment of the host during invasion and systemic spreading; however, the underlying mechanisms are not well understood.^{32,34} The gene expression of some extracellular aspartyl proteases of *C. glabrata* has only been studied in a mouse model and macrophages.¹⁴ The vacuolar protease gene expression under different physiological conditions (including nutrition) has not yet been reported. In this study, we describe the biochemical activity and regulation of various vacuolar proteases of *C. glabrata* in order to infer the potential functional role of each of these enzymes.

Materials and methods

Strains and growth conditions

The CBS138 strain of *C. glabrata* used in this study was kindly provided by Dr. Bernard Dujon from Institut Pasteur, France.⁷ The yeast strain was stored at -70°C in 30% glycerol. Cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) at 37°C for 48 h with constant shaking, and then inoculated into fresh YPD medium.

Culture media used to measure expression of putative genes coding for proteases

Several media were tested to determine the effects on *C. glabrata* genes coding for protease expression. The *C. glabrata* cells were harvested from YPD medium during early stationary phase (15 h) and then washed twice with minimal medium consisting of 0.17% yeast nitrogen base (YNB) without amino acids or ammonium sulfate. The nonproliferating cultures were incubated at 37°C for 6 h with constant shaking in YPD medium and in 0.17% YNB supplemented

with 2% glucose (YNBg) under five different nitrogen sources (either no nitrogen source, 0.5% ammonium sulfate, 0.2% bovine serum albumin (BSA), 2% proline, 2% peptone).

Preparation of crude extracts

Cultures in early stationary phase were harvested by centrifugation at $5000 \times g$ at 4°C for 10 min. Biomass cells were fragmented in a FAST Prep-24 using glass beads (7.5 g of glass beads, 12 ml of 0.1 M Tris-HCl at pH 7.5, and 5 g of cells) and pulses ($3 \times 20\text{ s}$ at 6.5 m/s with 2 min intervals on ice). Broken cells were centrifuged at $5000 \times g$ at 4°C for 10 min. The crude extract was carefully removed from the glass beads and centrifuged at $23,000 \times g$ at 4°C for 10 min. The supernatant was removed and centrifuged at $100,000 \times g$ at 4°C for 1.5 h using a Beckman ultracentrifuge. The corresponding soluble fraction or cell free extract and membrane fraction were used for enzyme assays and protein determination.

Isolation of intact vacuoles by density gradient

Isolation of intact vacuoles was carried out by modifying previously described procedures.^{26,33,41} Briefly, cells were harvested at early stationary phase of incubation by centrifugation at $2800 \times g$ for 5 min. They were then washed and resuspended in SOB buffer [1.2 M sorbitol, 5 mM dithiothreitol (DTT), 50 mM Tris pH 7.6] containing Zymolyase 20T (2 mg g^{-1} cells). For the liberation of vacuoles after 2 h of digestion at 30°C , the spheroplasts were pelleted by centrifugation ($3000 \times g$ for 10 min), resuspended in lysis buffer (1.1 M glycerol, 50 mM Tris at pH 7.6, and 1 mM DTT), and disrupted by a Potter Elvehjem homogenizer in an ice bath. Unbroken cells were eliminated by centrifugation at $3000 \times g$ for 10 min, and the supernatant was centrifuged at $54,000 \times g$ for 30 min. The vacuolar pellet was resuspended until a final concentration of 2% sucrose was reached using 15% sucrose and sucrose buffer (10 mM Tris pH 7.6, 50 mM glycerol, 125 mM KCl, 1 mM DTT) in order to form a density gradient with sucrose at 15%, 35% and 45% using the same buffer. The sample was then centrifuged at $54,000 \times g$ for 40 min. The interface (between 15% and 35%) containing the vacuoles was resuspended in solution E (0.6 M sorbitol, 5 mM Tris at pH 7.6, and 1 mM DTT) to a 1:4 proportion and stored at -80°C .

Fluorescence microscopy (FM)

When cells were resuspended in SOB buffer, vacuoles were stained adding two markers of the Yeast Vacuole Marker Sampler Kit (Molecular Probes; Life technology, USA). Cell Tracker blue CMAC (7-amino-4-chloromethyl-coumarin) was used to selectively stain the yeast vacuole lumen and a green fluorescent marker (MDY-64) to stain the yeast vacuole membrane. The markers were observed in a fluorescent microscope Imager.M2 (Zeiss, Germany) coupled to the Axio Vision program.

Vacuolar marker enzyme assays

Enzymatic activity was determined using previously described methods.^{11,12} The following substrates were used for the different proteases: denatured acid hemoglobin for proteinase A activity (PrA), hide powder azure (HPA) for proteinase B activity (PrB), and N-benzoyl-tyrosine-4-nitroanilide (N-BZ-Tyr-4-NA) for carboxypeptidase activity (CpY).²² Protein determinations were performed based on the Lowry method.²¹

Effect of protease inhibitors

The effect of inhibitors on protease activity was measured by previously described methods.²² The inhibitors bestatin (100 and 250 μ M), leupeptin (20 and 50 μ M), pepabloc (1 and 5 μ M), pepstatin (2 and 5 μ M), E-64 (10 and 50 μ M), 1–10 phenanthroline (1 and 10 mM), PMSF (1 and 5 mM), and EDTA (1 and 10 mM), were employed in this study.

Bioinformatics analysis of canonical vacuolar proteases

The sequences of putative genes encoding vacuolar proteases of *C. glabrata* were identified by BLAST analysis (<http://blast.ncbi.nlm.nih.gov> and <http://www.ensembl.org/Multi/blastview>). The putative orthologous genes from *S. cerevisiae* were used as templates in the *C. glabrata* genome project, available at <http://ncbi.labri.fr/Genolevures/elt/CAGL>. Promoter sequences were analyzed using the MatInspector database (<http://www.genomatix.de/>). Protein sequences were deduced from genes using the alternative yeast nuclear genetic code (available at <http://www.uniprot.org>). These databases contain protein sequences and functional information, as well as cross-references with other databases that display motifs or subcellular location predictions (<http://merops.sanger.ac.uk>). PCR oligonucleotides were designed by the Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0>).

RNA extraction

After 6 h of exposure to the respective media as described above, cells were harvested by centrifugation and frozen immediately. Frozen pellets were washed in diethylpyrocarbonate (DEPC)-treated water and resuspended in RNA isolation buffer (2% Triton, 1% SDS, 10 mM NaCl, 1 mM EDTA, and 10 mM Tris at pH 8.0 in DEPC water). Glass beads were added to constitute 2/3 of the total volume and total RNA was extracted by shaking (4×10 s). RNA extraction was performed using the acid phenol method. DNA was removed by treating the RNA with DNase I (Invitrogen, CA, USA), according to the manufacturer's instructions.

Primers and RT-PCR

To amplify fragments of *C. glabrata* genes encoding vacuolar proteases, specific primer pairs were designed using the gene sequences available at the *C. glabrata* genome database (<http://ncbi.labri.fr/Genolevures/elt/CAGL>): *CgPEP4* (F: 5' 691-TATCTGAAGAGTGTC AATGACCCAGC-716 3', R: 5' 1208-TACAGCCTCAGCTAACTGACAACATTGG-1236 3'); *CgPRB1* (F: 5' 30-AAGAAAGGCGTCTCTCCGGAGG-53 3', R: 5' 547-CGTA GTGTTTGAAGCGATGCTACC-571 3'); and *CgCPY1* (F: 5' 334-ATGACCC TGTCA TCCTATGGTTGAACGG-401 3', R: 5' 841-ACTGGGTCAGCACCATTCTTCACC-866 3'). The RT reaction for cDNA synthesis was performed using 18S rRNA primers: *Cg18S rDNA* (F: 5' 543-CAATTGGAGGGCAAGTCTGG-564 3', R: 5' 1267-TAAGAA CGGCCATGCACCAC-1286 3'). The reaction mixture was brought up to a final volume of 20 μ l, containing 2 μ g of

RNA and 100 U Superscript II Reverse Transcriptase (Invitrogen). RT-PCR determinations were performed in duplicate using the reaction conditions described by Loaiza-Loeza.²⁰ Gene expression was normalized according to levels of 18S rRNA expression. The intensity of each amplified band was measured with SigmaGel v.1 (Jandel Scientific Software USA) to obtain the *CgProtease*/18S rRNA ratio. The standard deviation of each treatment was estimated.

Results

Sequence analysis of *C. glabrata* putative genes encoding vacuolar proteases

A BLAST analysis based on the *C. glabrata* genome revealed the presence of five genes likely to encode vacuolar proteases, orthologous to *S. cerevisiae* genes: *CgPEP4* gene coding for aspartyl protease A (PrA), three *CgPRB* ORFs coding for serine protease (PrB) in three different locus (*CgPRB1*, *CgPRB2* and *CgPRB3*), and the *CgCPY1* gene coding for carboxypeptidase Y (CpY) (Table 1). The bioinformatics analysis suggest that the vacuole could be a putative site of subcellular location of the five predicted proteins, with a certainty factor of 0.9. The deduced proteins from the *CgPEP4*, *CgPRB1-3*, and *CgCPY1* genes showed a high percentage of similarity with deduced proteins from orthologous genes of *S. cerevisiae*. The molecular masses (Mr) ranged from 45.4 to 63.6 kDa (Table 1).

The analysis of the active site of the PrA protein of *C. glabrata* encoded by the *CgPEP4* gene displayed the catalytic domain DD (Asp and Asp), like *S. cerevisiae* PrA. This catalytic domain is characteristic of aspartyl proteases (Asp¹⁰⁹ and Asp²⁹⁴ in *S. cerevisiae*, and Asp¹⁰⁹ and Asp³⁰¹ in *C. glabrata*) (Fig. 1).

The deduced amino acid sequence alignment of *ScPRB1* and *CgPRB1-3* shows the serine protease DHS catalytic triad, which in *S. cerevisiae* is Asp³²⁵, His³⁵⁷ and Ser⁵¹⁹. In *C. glabrata* this triad is distributed in different positions in the amino acid sequence of the three proteases PrB [PrB1 (Asp¹⁴⁵, His¹⁷⁷ and Ser³³⁹), PrB2 (Asp²¹⁷, His²⁴⁹ and Ser⁴¹⁰) and PrB3 (Asp²¹⁵, His²⁴⁷ and Ser⁴¹¹)] (Fig. 1). On the other hand, the carboxypeptidase in *S. cerevisiae* and *C. glabrata* showed the serine protease SDH catalytic triad, which in *S. cerevisiae* is Ser²⁵⁷, Asp⁴⁴⁹ and His⁵⁰⁸, and in *C. glabrata* is Ser²³¹, Asp⁴²⁶ and His⁴⁸⁵ (Fig. 1).

In order to predict whether the three *CgPRB1-3* genes could be differentially expressed, an analysis of their promoter regions (4000 pb) was performed. These were compared to the promoter region of *PRB1* of *S. cerevisiae*. Differences were observed in the type and number of the promoter sequences for each *PRB* genes; likewise, promoter sequences that activate the regulatory genes of nitrogen YNIT (classification according to the nomenclature of the database, MatInspector) were identified in each of the three *PRB* genes. The *CgPRB1* and *CgPRB2* genes showed promoter sequences (GATA and URS1, respectively) for the regulation/repression of multiple metabolic pathways of nitrogen. Interestingly, *CgPRB1* presented 119 promoter sequences related to the synthesis of amino acids, the regulation of the cellular cycle, resistance to antibiotics, the regulation of pH response, heat shock, and the activation of multi-stress and oxidative stress. Meanwhile, *CgPRB2* only showed 6 sequences related to an ARS sequence and transcription factors, and one related to the regulation of metals. Moreover, *CgPRB3* has 57 promoter sequences, some of which are shared with the *CgPRB1* gene.

Assessment of vacuolar purity and integrity

Intact vacuoles from *C. glabrata* CBS138 were purified using the procedure described in the Material and Methods section. Fig. 2 shows the location of the vacuoles stained with MDY-64 and CMAC

Table 1*In silico* analysis of vacuolar proteases from *Candida glabrata*.

Protein/gene	Entry name	Sequence length AA	Mass (kDa)	Theoretical function	% Identity with <i>S. cerevisiae</i>	Gene names (EMBL)
PrA/Cg PEP4	Q6FK02.CANGA UniProt/TrEMBL	415	45.4	Aspartyl protease	62.0	CAGL0M02211g
<i>Cg PRB1</i>	Q6FX16.CANGA	468	50.0	Serine protease	65.0	CAGL0B03619g
PrB/Cg PRB2	Q6FVE0.CANGA	485	53.4	Serine protease	53.0	CAGL0E02651g
<i>Cg PRB3</i>	Q6FK98.CANGA	579	63.6	Serine protease	39.0	CAGL0L13266g
CpY/CgCPY	Q6FIK7.CANGA UniProt/TrEMBL	508	57.2	Serine type carboxypeptidase activity	74.0	CAGL0M13651g

PrA = Proteinase A; PrB = Proteinase B and CpY = Carboxypeptidase Y.

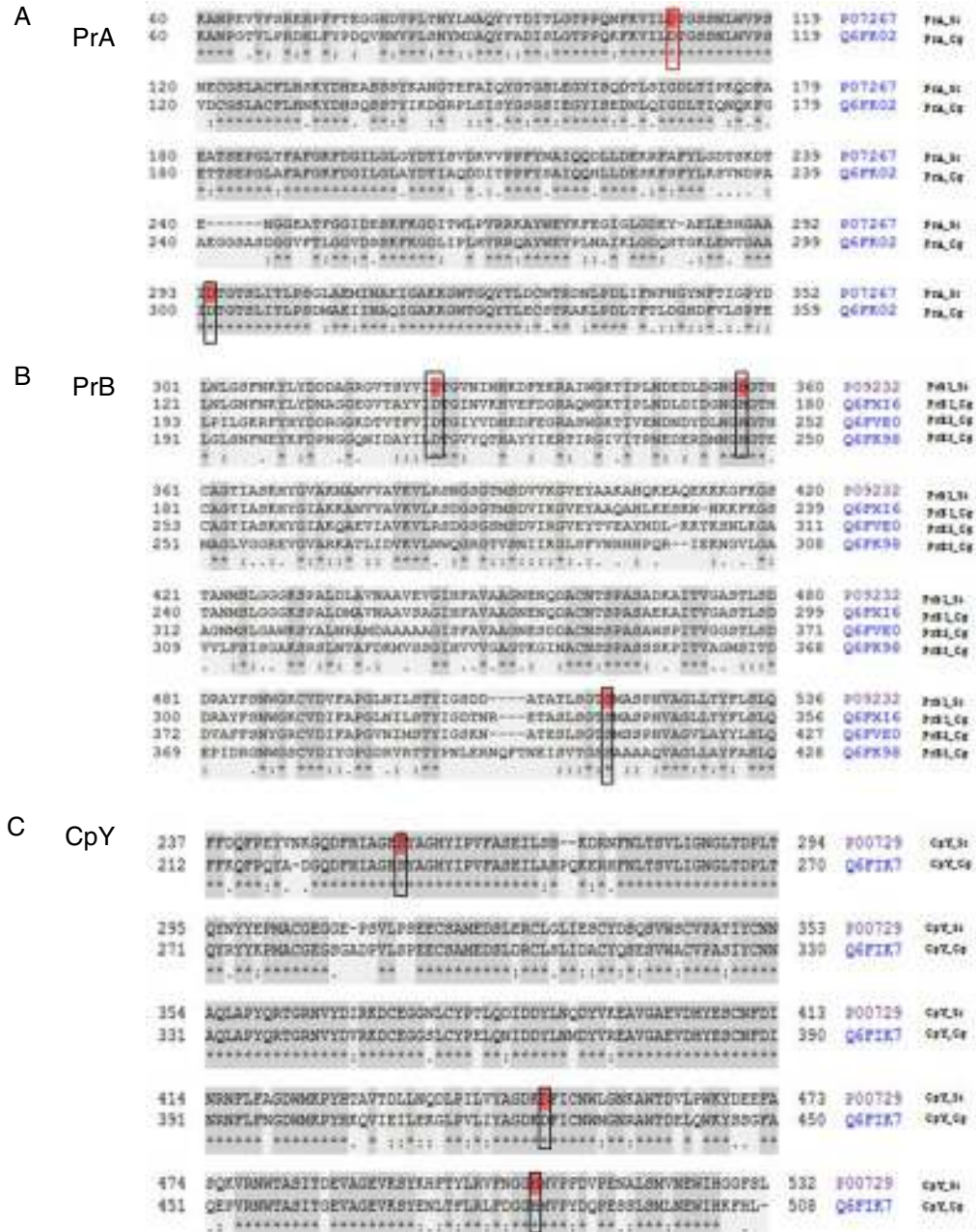


Fig. 1. Peptide sequence alignment of vacuolar proteases. (A) Proteinase A of *S. cerevisiae* and of *C. glabrata* in the active DD frame site (Asp¹⁰⁹ and Asp²⁹⁴ in *S. cerevisiae*; Asp¹⁰⁹ and Asp³⁰¹ in *C. glabrata*). (B) Proteinase B of *S. cerevisiae* and three putative sequences of *C. glabrata*. These four sequences show the DHS catalytic triad (Asp³²⁵, His³⁵⁷, and Ser⁵¹⁹ in *S. cerevisiae*; Asp¹⁴⁵, His¹⁷⁷, and Ser³³⁹ in PrB1 of *C. glabrata*; Asp²¹⁷, His²⁴⁹, and Ser⁴¹⁰ in PrB2 of *C. glabrata*; Asp²¹⁵, His²⁴⁷, and Ser⁴¹¹ in PrB3 of *C. glabrata*). (C) Carboxypeptidase in *S. cerevisiae* and *C. glabrata* identified in the SDH catalytic triad framed: Ser²⁵⁷, Asp⁴⁴⁹, and His⁵⁰⁸ in *S. cerevisiae*; Ser²³¹, Asp⁴²⁶, and His⁴⁸⁵ in *C. glabrata* (<http://www.uniprot.org/>).

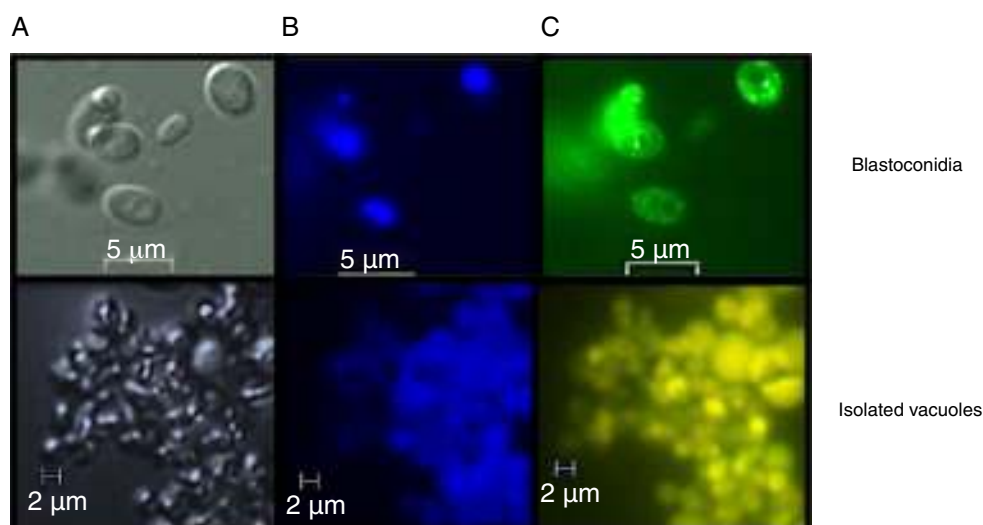


Fig. 2. Visualization of *C. glabrata* vacuoles by FM. (A) Cells observed by differential interference contrast (DIC) microscopy without staining. (B) Vacuolar lumen stained with CMAC (7-amino-4-chloromethyl coumarin) and (C) with the yeast vacuole membrane marker MDY-64.

within the *C. glabrata* blastoconidia. The micrographs obtained by FM confirmed the vacuole integrity after the purification process and showed vacuoles clusters of irregular size.

The enzymatic activity, location and inhibition of PrA, PrB, and CpY

In *C. glabrata*, intracellular activity of PrA, PrB, and CpY was found. These different kinds of proteolytic activity were evidenced during the early stationary phase in YPD medium (15 h) (data not shown). These proteases from *C. glabrata* were found in the soluble (cell-free extract) and vacuolar fraction (Table 2). The enzymatic activity of PrA, PrB and CpY from purified vacuoles yielded enrichment factors of 37, 6.7- and 7-fold, respectively, compared to spheroplasts (Table 2).

To establish the proteases types, eight protease inhibitors were applied to the vacuolar fraction from *C. glabrata*. Pepstatin A (2 mM), a specific inhibitor of aspartic proteases, showed inhibition of PrA residual activity (10%). Serine protease inhibitors, such as Pefabloc and PMSF (both at 5 mM), caused inhibition of 20% and 12% of PrB, respectively. Pefabloc (5 mM) and PMSF (1 mM) caused strong inhibition of the residual activity of CpY, 12% and 3%, respectively. Chelating agents, such as EDTA (10 mM) and 1–10 phenanthroline (10 mM), did not exert a clear inhibitory effect, suggesting that these proteases are not metalloenzymes. Leupeptin (1 mM) and E-64 (5 mM) did not show significant inhibitory activity, which rules out the presence of cysteine groups in the catalytic domain of the proteases. The lack of inhibitory effect of bestatin excludes the presence of aminopeptidases (data not shown).

The presence of endogenous inhibitors of vacuolar proteases has been reported for *S. cerevisiae*.¹⁸ Since they could also exist in *C. glabrata*, the determination of enzymatic activity was conducted in conditions that inactivate these inhibitors. Accordingly and in order to evaluate PrA activity, this enzyme was preincubated at pH 5. PrB activity was assessed with the addition of SDS, and CPY activity by adding sodium deoxycholate.¹²

Proteolytic activity of putative vacuolar proteases under different physiological conditions

The nitrogen source plays an important role in regulating the expression of protease-encoding genes in different organisms. Therefore, the expression can be measured at the level of enzyme

activity or mRNA. To determine whether proteolytic activity is affected at the protein expression level by the nitrogen source, *C. glabrata* was cultured in YPD at early stationary phase (15 h) and then tested in media with and without a nitrogen source. Accordingly, YNBg medium was supplemented with different nitrogen sources: ammonium, proline, peptone, and BSA. The level of protease activity was analyzed at 3 and 6 h. The level of enzymatic specific activity of the three proteases studied showed little variation when making the transition from YPD to a different medium. Only the enzymatic activity of PrA and CpY increased (more than two-fold) in the presence of ammonium (basal value at 0 h versus the value at 6 h). Furthermore, with this nitrogen source PrA and CpY reached their maximum activity (Table 3).

Expression of CgPEP4, CgPRB1 and CgCPY1 genes in different nitrogen sources

The regulation of expression of putative gene-encoding proteases in *C. glabrata* by distinct sources of nitrogen and under conditions of nutritional stress (nitrogen starvation) in several culture media at 6 h was evaluated by RT-PCR (Fig. 3). Under the conditions studied, the *CgPEP4*, *CgPRB1*, and *CgCPY1* genes were expressed in YNBg medium with ammonium as the nitrogen source. *CgPRB1* was the only gene that was expressed with both, BSA as a nitrogen source and without a nitrogen source. Moreover, this gene also showed expression with ammonium and peptone; however, none of these genes were expressed in the YNBg medium with proline as the nitrogen source. The expression of genes in the YPD medium was not very evident, which is consistent with the levels of the specific activity found (Table 3).

Discussion

Fungal vacuoles have been recognized as organelles that are involved in protein turnover, cellular homeostasis and membrane trafficking, and are essential for many of the attributes that define the functioning of fungi in their natural environments.⁴²

C. glabrata displayed proteolytic activity similar to that found in some other yeasts, including *S. cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, and *Yarrowia lipolytica*.^{9,10,24,37,38} Previous data revealed that the levels of PrA, PrB, and CpY proteases are dependent on the growth phase and composition of the nutrients in the growth media, e.g. higher levels of these proteases

Table 2Enrichment of the specific activity of vacuolar marker enzymes in intact vacuoles isolated from *C. glabrata*.

Protease	PrA		PrB		CpY	
Cell fraction	Specific activity mU mg ⁻¹	Enrichment (fold)	Specific activity mU mg ⁻¹	Enrichment (fold)	Specific activity mU mg ⁻¹	Enrichment (fold)
Spheroplast	0.5	1.00	7.2	1.0	0.9	1.0
Cell-free extract	1.7	3.4	8.9	1.2	1.4	1.6
Vacuolar fraction	18.7	37.4	48.0	6.7	6.4	7.1

PrA = Proteinase A; PrB = Proteinase B and CpY = Carboxypeptidase Y.

Table 3Specific activity of PrA, PrB and CpY from *C. glabrata*, determined from the cell free extract. The yeast was cultivated in YNB medium with different sources of nitrogen.

Medium	Exposure time (h)	Protease		
		PrA	PrB	CpY
		Specific activity		
		mU mg ⁻¹		U mg ⁻¹
YNBg	0	0.20	0.18	8.56
	3	0.63	0.28	9.14
	6	0.54	0.25	10.16
YNBg + NH ₄ 0.5%	0	0.27	0.79	9.91
	3	0.59	0.34	16.97
	6	0.62	0.58	22.12
YNBg + Proline 2.0%	0	0.30	0.20	7.46
	3	0.51	0.25	5.30
	6	0.44	0.17	7.28
YNBg + Peptone 2%	0	0.24	0.75	11.09
	3	0.30	0.27	9.65
	6	0.20	0.30	11.32
YNBg + BSA 0.2%	0	0.26	0.76	8.37
	3	0.23	0.44	8.19
	6	0.21	0.49	9.49
YPD	0	0.25	0.48	7.87
	3	0.30	0.17	6.26
	6	0.15	0.24	5.65

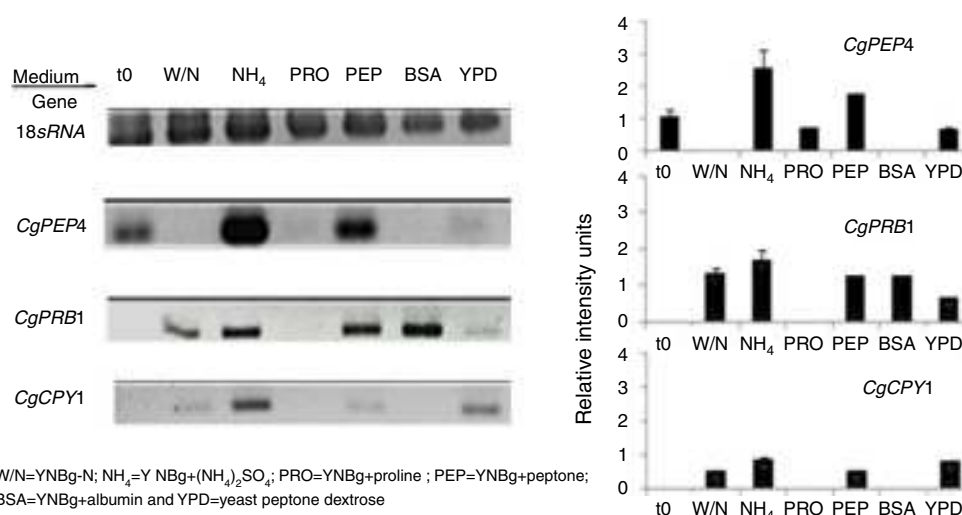
PrA = Proteinase A; PrB = Proteinase B and CpY = Carboxypeptidase Y.

YNBg = medium containing yeast nitrogen base with 2% glucose.

detected during early stationary phase in this study (data not shown). These results suggest that these proteases might be regulated by the source of nitrogen and carbon, as occurs with other fungi.^{9,10,37,38} Our findings based on the enzymatic activity of PrA, PrB and CpY proteases from the vacuolar fraction of *C. glabrata* displayed an increased activity suggesting their specific location in this organelle. The value of the enriched-activity yield for PrA is commensurate with the preparation of the pure vacuole as described

in *S. cerevisiae*.³³ Proteases that are considered canonical *S. cerevisiae* vacuolar enzymes could be used as vacuolar markers for *C. glabrata*.³³

The regulation of the *CgPEP4*, *CgPRB1* and *CgCPY1* genes at the transcription level does not correspond completely to the levels of activity found for the enzymes coded by these genes. This may be due to post-translational processing of vacuolar proteases in this organelle, as described in *S. cerevisiae* or *S. pombe*.^{1,30,39}

**Fig. 3.** Expression of the *CgPEP4*, *CgPRB1* and *CgCPY1* genes in culture media with different sources of nitrogen determined by RT-PCR.

In *C. albicans*, it has been reported that the source of nitrogen and phase of growth influence the level of expression of vacuolar aspartic protease Apr1p (orthologous of PrA) and carboxypeptidase Cpy1p.³ On the other hand, it has been described that yeasts preferentially utilize glutamine, asparagine and ammonium as nitrogen sources, while proline and glutamate are poor sources of nitrogen.²⁵ In the present study a differential expression was observed, according to the source of nitrogen employed in the three genes studied (*CgPEP4*, *CgPRB1*, and *CgCPY*); however, no expression of these three genes was found when they were tested in the presence of proline. Contrarily, an overexpression in *C. albicans* of dipeptidyl aminopeptidase, encoded by the *CaSTE13* gene, was observed when using proline as the source of nitrogen.⁴ Intracellular proteolytic activity is very important for the protein turnover in *S. cerevisiae*, and may have the same function in *C. glabrata*.⁶

In this work, an acidic aspartyl proteinase (PrA) was detected in the *C. glabrata* vacuole, and the specific inhibitor Pepstatin A inhibited its activity. In *S. cerevisiae*, the *ScPEP4* gene codes for an aspartyl protease, called proteinase A (Pep4) that is responsible for the sequential activation of many vacuolar hydrolases such as proteinase B and carboxypeptidase Y.¹³ The deletion of the *ScPEP4* gene induces a reduction of the hydrolytic ability of the vacuole in *S. cerevisiae*. However, no detectable phenotypic consequences have been described in the *C. albicans pep4Δ* mutant.²⁸ Hence, there may be significant differences between the proteolytic systems that operate in the vacuoles of *C. albicans* and the *S. cerevisiae*. Bioinformatics analysis in the *C. glabrata* genome showed only one putative *CgPEP4* gene encoding for an acidic aspartyl proteinase located in a vacuole. The protein deduced from the *CgPEP4* gene has a theoretical molecular mass similar to PrA of *S. cerevisiae* (45.4 kDa for PrAcg and 44.5 kDa for PrAsc). Moreover, a DD amino acid sequence of the catalytic domain confirms that the *CgPEP4* gene codes for an aspartyl protease. The highest levels of mRNA expression of the *CgPEP4* gene occurred in the presence of ammonium and peptone as sources of nitrogen. Contrarily, the absence of a nitrogen source did not cause an increase in the expression of this gene. The increase in *CgPEP4* gene expression observed in culture media with ammonium or peptone as a nitrogen source may be due to the presence of GATA promoter sequences that exert physiological control over the catabolism of nitrogen (NCR) by two pathways, activation or repression.⁶ The *CgPEP4* promoter sequence showed 21 putative binding sites for GATA proteins, while the *ScPEP4* promoter sequence showed only one binding site for this protein. Coffman and Cooper suggest that global regulatory mechanisms are stimulated by nitrogen from distinct sources, including small molecules such as ammonium and macromolecules like peptone.⁶

Proteinase B activity was detected using HPA as a substrate, which resulted in a serine protease. In *S. cerevisiae*, proteinase B is involved in protein degradation in the vacuoles and is required for full protein degradation during sporulation.³⁷ Therefore, PrB may be responsible for the activation of other vacuolar proteins in *C. glabrata*, and could participate in yeast turnover. Three ORFs coding for hypothetical PrB (*Cg PRB1-3*) were identified in the *C. glabrata* genome; meanwhile, in the *S. cerevisiae* genome only one ORF was found for this gene encoding for PrB. The proteins deduced from the *CgPRB1-3* genes have a theoretical molecular mass from 50 to 63.6 kDa that are homologous to the PrB protein of 69 kDa in *S. cerevisiae*. It has been suggested that gene duplications in *C. glabrata* might be important adaptations for the success of this opportunistic pathogen. These duplications include the coding for glycosylphosphatidylinositol-linked aspartyl proteases by *YPS* genes, and for glycosylphosphatidylinositol-linked epithelial adhesins by *EPA* genes.^{7,31} The variation in the promoter sequences in each of the putative *CgPRB* genes suggests a differential expression between the three genes herein studied. The expression of the *CgPRB1* gene would be regulated mainly by distinct sources

of nitrogen, environmental changes, pH, and temperature. On the other hand, the *CgPRB2* gene has a promoter sequence that responds to the beginning of cellular replication, while the *CgPRB3* gene has a promoter sequence that is active during the cellular cycle. The duplication of *CgPRB* genes coding for PrB and the diversity of promoter sequences within each gene might provide *C. glabrata* with survival advantages under certain environmental conditions. Accordingly, it would be interesting to study the possible duplication of the *CgPRB* gene in *C. glabrata*.³¹ Hence, the generation of a mutant in the *CgPRB* gene could clarify the role of the PrB protein in the life cycle of the yeast.

In this study, BSA was also employed as a nitrogen source in order to evaluate the expression of *CgPEP4*, *CgPRB1*, and *CgCPY* genes. Of the three genes herein studied, only *CgPRB1* showed an increased expression in BSA. Although PrB is a protein with vacuolar location, it has been suggested that it participates in the protein turnover that helps the yeast survive. On the other hand, it has been demonstrated that other proteases, such as the aspartyl protease secreted by Sap2 of *C. albicans* and *C. dubliniensis*, induce the expression of PrB in the presence of BSA.^{20,25} The activity of the PrB protease may support the adaptation and survival of *C. glabrata* in the host, especially during systemic infections.³⁵

In the present study, results performed with the CpY protease of *C. glabrata* displayed a serine protease activity in the soluble fraction. Besides, theoretical analysis of CpY protease showed a molecular mass of 57 kDa, while the homologue enzyme of *S. cerevisiae* has a molecular mass of 60 kDa. Upregulation of *CgCPY1* gene expression and activity occurred in the YNBg-ammonium medium. In *C. albicans*, the determination of *CaCPY1* gene expression under nitrogen-limited conditions revealed that this gene was regulated at both the transcriptional and translational levels.³ In *S. cerevisiae*, carboxypeptidase activity is involved in low-nitrogen conditions, sporulation, process of killing toxins, and non-specific protein degradation in the vacuole; in addition, carboxypeptidase Y is also considered a vacuolar marker enzyme.³³

C. glabrata can survive in nutrients-poor conditions (e.g. on the surface of epithelial cells and inert surfaces like catheters where the yeast can form biofilms).³⁶ Another characteristic of this yeast is its capacity to survive in macrophages, which implies that it can endure the respiratory burst.³⁵ Roetzer et al. demonstrated the formation of peroxisomes in yeasts and the activation of autophagy involved in their capacity of intracellular survival,³² although few studies have been conducted in this regard. There have been reports about the preponderant role of the yeast vacuole and its proteases during autophagy, assisting in the degradation and protein turnover under stressful conditions.^{16,43} Consequently, the study of vacuolar proteases herein described, as well as other proteases such as aminopeptidases, should allow researchers to elucidate the role played in the survival and proliferation of *C. glabrata* during the process of host infection. These results establish the basis for the biochemical and molecular study of vacuolar proteases in *C. glabrata*. The role of vacuole-mediated proteolysis in this yeast may be explored by obtaining the corresponding mutants and studying host-pathogen interactions.

Conflict of interest

The authors declare that they have no conflict of interests in relation to any of the techniques or materials used in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.riam.2014.10.005>.

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