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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.

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

Palabras clave:
Proteasas
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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

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y la hemoglobina ácida desnaturalizada; serín proteasa neutra, con actividad sobre el substrato 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.

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 × *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 × 20 s at 6.5 m/s with 2 min intervals on ice). Broken cells were centrifuged at 5000 × *g* at 4 °C for 10 min. The crude extract was carefully removed from the glass beads and centrifuged at 23,000 × *g* at 4 °C for 10 min. The supernatant was removed and centrifuged at 100,000 × *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 × *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 × *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 × *g* for 10 min, and the supernatant was centrifuged at 54,000 × *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 × *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.