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Biochemical study of intracellular proteases from the phytopathogenic fungus *Sporisorium reilianum*

Estudio bioquímico de proteasas intracelulares del hongo fitopatógeno *Sporisorium reilianum*

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Abstract

The intracellular proteolytic system of the phytopathogen *Sporisorium reilianum* is not well-understood. This work reports the presence of at least three intracellular proteases. The activity of dipeptidyl aminopeptidase (psrDAPi), carboxypeptidase (psrCPi) and proteinase A (psrPAi) were measured under several nutritional conditions. All enzymes were detected in the soluble and membrane fractions, and in membrane fractions. Zymographic analysis demonstrated that this fungus contains at least one soluble psrDAPi and two membranal isoforms. psrCPi was found to be regulated by an endogenous inhibitor. The effect of different proteases inhibitors was evaluated against the intracellular enzymes in study.

Head smut, Intracellular proteases, *Sporisorium Reilianum*

Resumen

El sistema proteolítico del hongo fitopatógeno *Sporisorium reilianum* no había sido estudiado. En este trabajo se reporta la presencia de al menos tres proteasas intracelulares. Las actividades de, dipeptidil aminopeptidasa (psrDAPi), carboxipeptidasa (psrCPi) and proteinasa A (psrPAi) fueron medidas bajo diferentes condiciones nutricionales. Se encontró que todas las enzimas fueron detectadas en la fracción soluble y asociadas a la membrana. El análisis zimográfico demostró que este hongo contiene al menos una isoforma soluble y dos en membranales. psrCPi se encuentra regulada por la presencia de un inhibidor endógeno. Se determinó el efecto de diferentes inhibidores de proteasas sobre las enzimas encontradas en este estudio.

Carbón de la espiga, Proteasas intracelulares, *Sporisorium Reilianum*

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Introduction

S. reilianum is the causal agent of head smut, a disease that affects corn worldwide. This basidiomycete infects corn plants during seed germination, but symptoms do not become visible until flowering time, with the presence of phyllody and carbonaceous masses of teliospores that invade the male inflorescences and cobs. These fungal structures are later released and can fall or be disseminated by wind or rain. Under optimal conditions, the teliospores geminate in the soil where they produce a basidium with four haploid basidiospores that bud in the same way as a yeast. In young plant tissues, two sexually-compatible basidiospores fuse to form an infective dikaryotic hypha that penetrates the plant's epidermis through an appressorium. Colonization is progressive and the mycelium can be observed in all plant tissues, though sporulation only occurs in the tassels and ears [1, 2, 3, 4].

Proteolysis is a vital process for cells due to the important functions that it performs, such as utilizing exogenous proteins as nutrients, eliminating non-functional proteins, maintaining amino acid pools, and post-translational control [5]. The survival of cells in their natural environment depends on their ability to adapt to frequent changes. Proteolysis plays an important role in responding to stress caused by nutrient starvation, variations in pH, temperature and UV radiation, and the presence of heavy metal ions or toxins. One example of proteolysis is spore formation in *Saccharomyces cerevisiae* when the carbon source available is poor. This process manifests high proteolytic activity that provides amino acids to the cells for new protein synthesis and energy generation [6].

S. cerevisiae has been employed as a study model to elucidate intracellular protease functions in eukaryotic cells, leading to the biochemical and genetic characterization of several of their proteolytic enzymes [5, 6, 7]. Intracellular proteolytic enzymes have also been identified in other yeasts of biotechnological interest, such as *Schizosaccharomyces pombe* and *Yarrowia lipolytica* [8, 9].

The intracellular proteolytic system of phytopathogenic fungi has only been reported for the basidiomycete *Ustilago maydis*, which produced the intracellular proteinases pumA and pumB, as well as aminopeptidase pumAPE and dipeptidyl aminopeptidase pumDAP [10]. In this fungus, the *pep4* gene that encodes a vacuolar proteinase, A, is involved in dimorphism and pathogenesis [11]. Also, the X-prolyl-dipeptidyl aminopeptidase encoded by *dapUm* gene has been cloned and transformed in *Pichia pastoris* for pharmaceutical purposes [12]. However, the intracellular proteolytic system of *S. reilianum* remains largely unknown. The study of its proteases localized in the interior of the cell will contribute to increasing our knowledge of this phytopathogenic fungus.

Materials and Methods

Throughout this study, the intracellular proteases from *S. reilianum* are identified by the prefix "psr" (for protease *S. reilianum*) while "i" indicates intracellular activity.

Microorganism and culture conditions

The *S. reilianum* diploid strain was provided by Dr. Santos Gerardo Leyva Myr of the Universidad Autónoma Chapingo, Mexico. It was isolated from corn crops in the state of Hidalgo in central Mexico. *Saccharomyces cerevisiae* DBY was used as the reference strain in this study. It was kindly provided by Dr. María Paz Suárez Rendueles of the Universidad de Oviedo, Spain. The strains were routinely maintained on YEPD plates (0.1% yeast extract, 2% peptone, 2% glucose, 2% agar) at 28°C. *S. reilianum* was conserved in inclined tubes with the same medium and mineral oil at room temperature, while *S. cerevisiae* stored in glycerol at 25% at -70°C.

S. reilianum was grown in liquid cultures in YEPD with minimal medium (0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate and 2% Glucose) [8] and different sources of nitrogen: 1% peptone, 0.5% proline, 0.5% ammonium sulfate or corn infusion (4 mg of protein/mL of medium). The corn infusion was obtained as described Mercado-Flores et al. [10].

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Production of intracellular proteases

The production of intracellular proteases was carried out in 250-mL Erlenmeyer flasks containing 25 mL of either of either media just described. A pre-inoculum of *S. reilianum* was prepared in each medium and incubated for 24 h at 28°C at 150 rpm, and then used to inoculate each flask until all were adjusted to a final absorbance of 0.2 at 600 nm. All cultures were incubated at 28°C at 150 rpm. Three flasks were taken as samples at 0, 6, 12, 24, 48, 72, 96 and 120 h.

The Enzymatic Crude Extract (ECE) was obtained as follows: the culture from each flask was centrifuged at 5,000 g at 4°C for 10 min. The biomass was collected, washed twice with distilled water and then transferred to a rupture mechanical in Vortex. The mixture contained 7.5g of glass beads (0.5 mm in diameter), 12.5 mL of 0.1 M Tris-HCl pH 7.5, and 5 g of biomass. Total cellular disintegration time was 20 min, vortexing lasted 1 min, and the mixture was then placed in ice for 1 min. The lysate was removed from the glass beads and centrifuged at 10,000 g at 4°C for 10 min. The supernatant (ECE) was collected and used to determine proteolytic activity. Culture growth was followed by absorbance at 600 nm.

Enzymatic assays and protein determination

In-plate testing to determine the intracellular presence dipeptidyl aminopeptidase (psrDAPi) was performed in four plates with YEPD, where *S. reilianum* and *S. cerevisiae* DBY were inoculated by closed stripe each one in the middle of the plate and incubated at 28°C for 48 h. The cultures in two plates were permeabilized with chloroform. The plates were used for each enzyme (two with permeabilized cellules, two with non-permeabilized cellules). The substrate lysyl-prolyl-ß-naphthylamide was used to determine psrDAPi. Their activity was revealed as described by Hirsh et al. [13].

The enzymatic activity of psrDAPi, carboxypeptidase (psrCPi) and proteinase A (psrPAi) were determined as described by Hirsh et al. [13]. The following substrates were used: L-alanyl-prolyl-4-nitroanilide for psrDAPi, N-benzoyl-tyr-4-nitroanilide for psrCPi, and acid-denatured hemoglobin for psrPAi.

To identify the presence of the endogenous inhibitor of psrCPi activity, the ECE was incubated with 50 µL of sodium deoxycholate (5%) at 37°C for 5 min, followed by the standard enzymatic assay [13]. Protein determinations were performed following Bradford [14] using bovine serum albumin as the standard.

Differential centrifugation, zymogram analysis and proteolytic inhibitor effect

To determine whether the proteolytic enzymes were soluble or associated with the membrane, the ECE obtained from the different culture media described above in which enzyme activity was highest were centrifuged at 100,000 g at 4°C for 1.5 h using a Beckman ultracentrifuge. The supernatant (soluble fraction) and precipitate (membrane fraction) were used as Enzymatic Extract to determine the different proteases. The membrane fraction was resuspended in 1 mL of Tris-HCl at 0.1M, pH 7.5. The enolase activity was used as intracellular marker [10]. psrDAPi activity was detected in situ using zymograms, as described by Suárez and Wolf [15]. The substrate lysyl-prolyl-ß-naphthylamide was used. The zymograms were incubated at 37°C until red bands appeared to indicate enzymatic activity.

The protease inhibitors Na_2EDTA, E64, 1-10 phenanthroline, bestatin, PMSF and pepstatin A were evaluated to ascertain their effect against the proteases studied. In this procedure, the ECE were pre-incubated with the respective inhibitor for 30 min at 37°C before conducting the standard enzymatic assay.

Results

In-plate testing of psrDAPi activity

The intracellular activity of psrDAPi was determined in plate. The enzyme was detected in permeabilized cellules, and also found in non-permeabilized cellules (Fig. 1).

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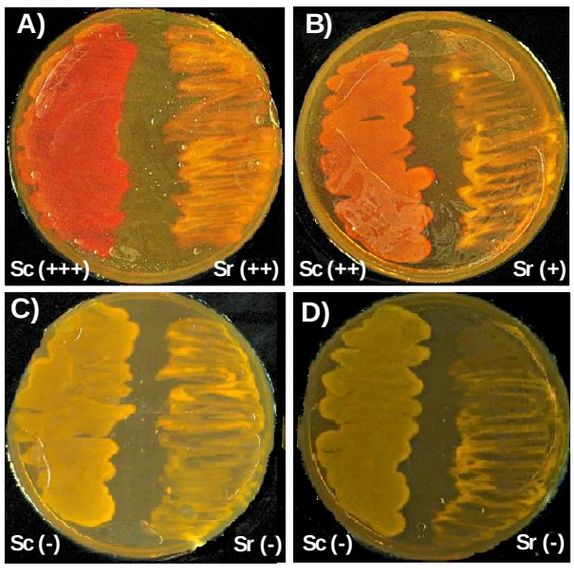
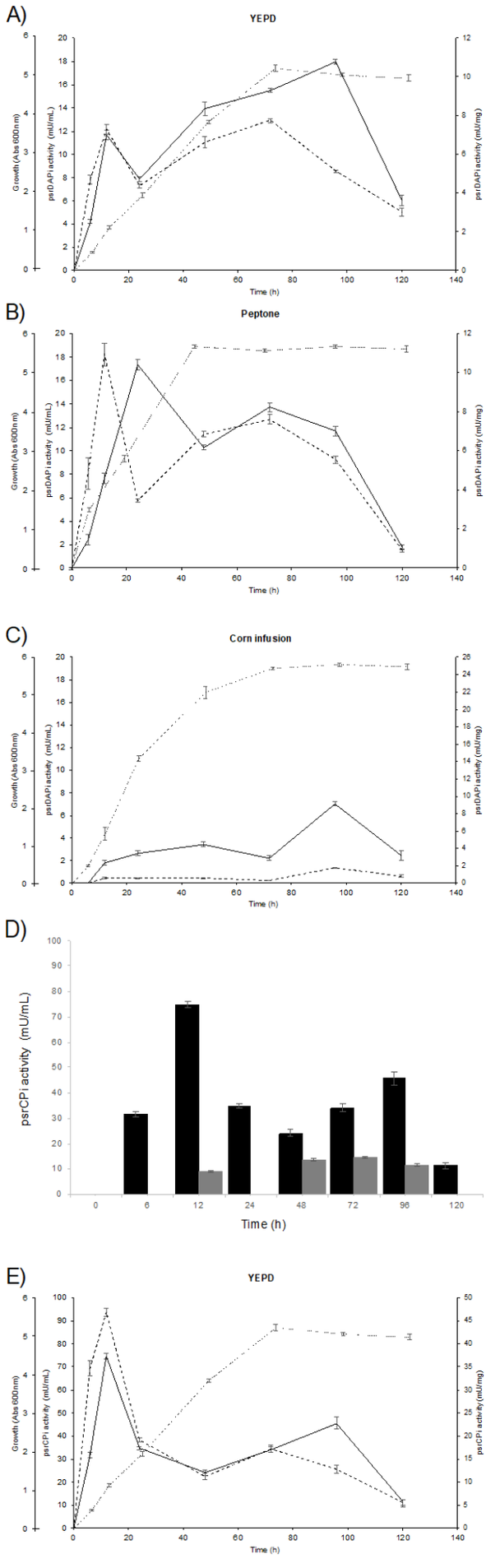


Figure 1 Plate test of psrDAPi activity. A) Activity in permeabilized cellules. B) Activity in non-permeabilized cellules. C) Control without substrate in permeabilized cellules. D) Control without substrate in non-permeabilized cellules. Sc= S. cerevisiae, Sr= S. reilianum. The symbols + and – indicate positive and negative tests, respectively. The cellules were permeabilized with chloroform as described in the Materials and Methods section.

Production of intracellular proteases of S. reilianum in different nitrogen sources

psrDAPi activity achieved its greatest activity in the YEPD medium and its minimal level with peptone, but was also detected in the minimal medium with the corn infusion (Fig. 3A-C). However, it was not found in the media that contained proline and ammonium sulfate.

In order to reveal psrCPi activity, it was necessary to add sodium deoxycholate, as shown in Figure 3D. This made it possible to determine its production in the YEPD and minimal peptone media, the corn infusion, and with ammonium sulfate. The highest activity levels were observed at 12h in YEPD and in the medium with ammonium sulfate (Fig. 3E-G). Meanwhile, psrPAi was found in all the culture media, reaching its highest activity levels in the YEPD medium at 48 h (Fig. 3H-L).



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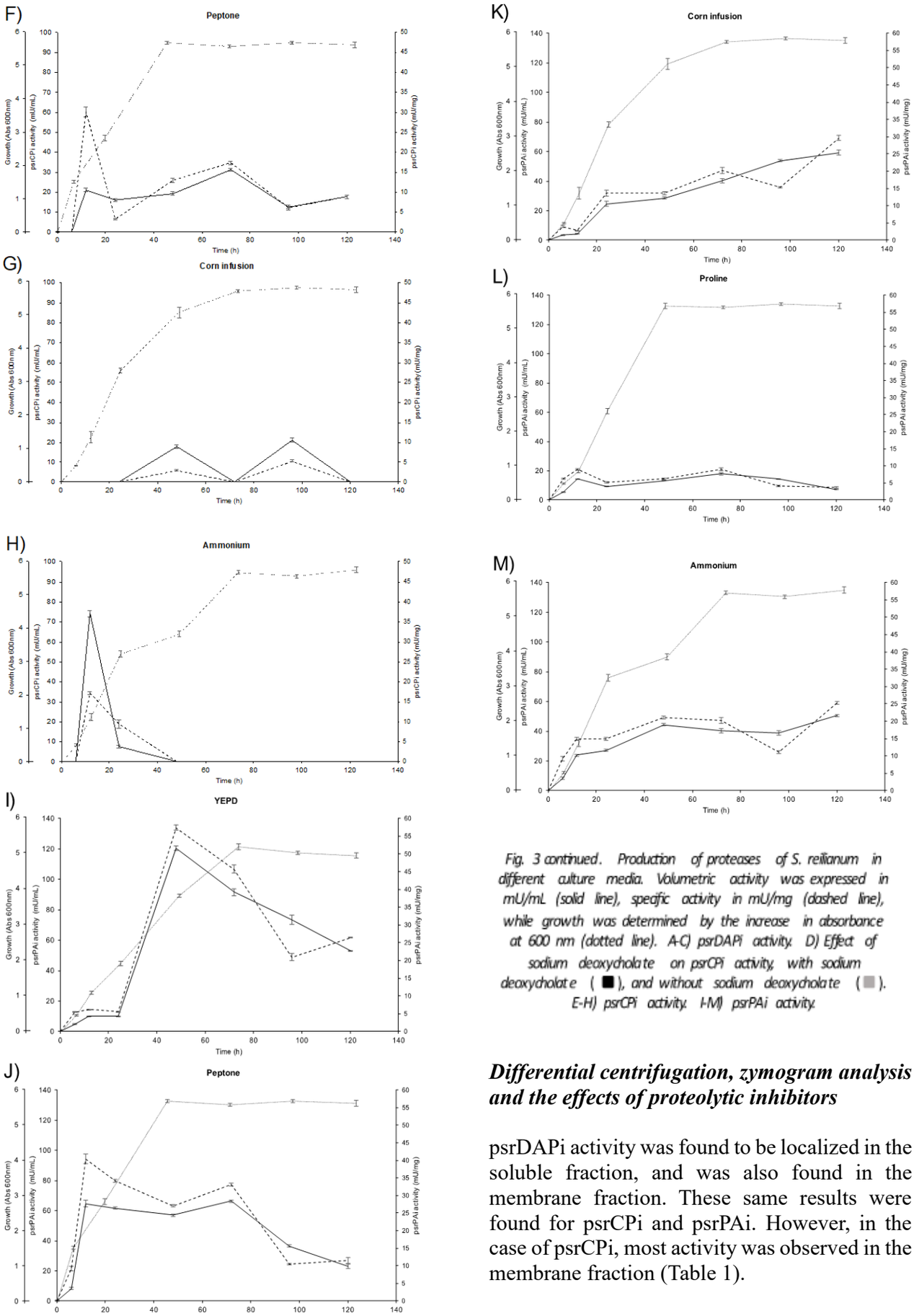

Fig. 3 continued. Production of proteases of S. reilianum in different culture media. Volumetric activity was expressed in mU/mL (solid line), specific activity in mU/mg (dashed line), while growth was determined by the increase in absorbance at 600 nm (dotted line). A-C) psrDAPi activity. D) Effect of sodium deoxycholate on psrCPi activity, with sodium deoxycholate (■), and without sodium deoxycholate (■). E-H) psrCPi activity. I-M) psrPAi activity.

Differential centrifugation, zymogram analysis and the effects of proteolytic inhibitors

psrDAPi activity was found to be localized in the soluble fraction, and was also found in the membrane fraction. These same results were found for psrCPi and psrPAi. However, in the case of psrCPi, most activity was observed in the membrane fraction (Table 1).

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Cellular fraction	Activity (%) psrDAPi	psrCPi	psrPAi	Enolase
ECE	100 ± 0.8	100 ± 0.5	100 ± 0.5	100 ± 2.4
Soluble fraction	63.7 ± 3.2	29.1 ± 0.4	73.0 ± 1.7	92.3 ± 4.4
Membrane fraction	35.6 ± 0.7	65.6 ± 0.7	24.6 ± 0.9	0.0

The enzymatic extracts were obtained from a culture of 96 h in YEPD

Table 1 Distribution of enzyme activities in cellular fractions of S. reilianum

Upon elaborating zymograms to reveal the isoenzyme profiles of the protease psrDAPi, the study found only one band of activity in the soluble fraction, but two bands of in the membrane fraction (Fig. 4).

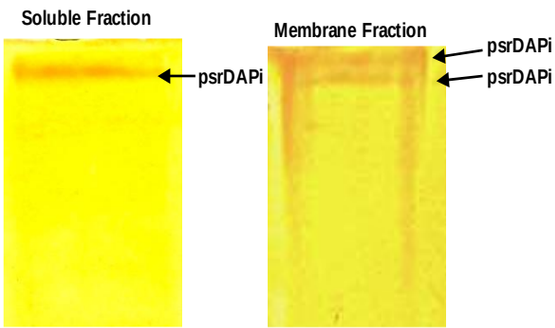

Figure 4 psrDAPi zymograms. The red bands indicate proteolytic activity

The evaluation of the effect of different protease inhibitors on the enzymes studied showed that the psrDAPi from the soluble fraction was only affected by E-64, in contrast to its activity in the membrane fraction, which was inhibited by PMSF. In the case of psrCPi activity in the soluble and membrane fractions, it decreased considerably upon adding PMSF, whereas the psrPAi from the same fractions was highly-affected by pepstatin A. Finally, the acid protease of the membrane fraction was also inhibited by PMSF, EDTA and E-64 (Table 2).

Discussion

The study of the biology of *S. reilianum* has been limited to determining its life cycle, mechanisms of pathogenicity, control, and some extracellular enzymes [2-4, 16, 17]. However, the intracellular proteolytic activity of this fungus had not been studied previously.

The present study found that *S. reilianum* produces psrDAPi. Similar activity has been reported for *U maydis* involving an enzyme called pumDAP, though there are observable differences in its production, since pumDAP is produced only in a minimal medium with proline and a corn infusion as the source of nitrogen [10], while psrDAPi is produced in YPD medium and a minimal medium with peptone, with low levels found in a medium with a corn infusion.

The best-characterized dipeptidyl aminopeptidases of fungal origin come from *S. cereviseae* and are called yscDAP A and yscDAP B. The first is located in the membrane of the Golgi apparatus and participates in processing the precursor of sexual factor α [18, 19]. yscDAP B, meanwhile, is found bonded to the vacuolar membrane.

Over-expression of yscDAP B in mutants that lack yscDAP A results in the maturation of sexual factor α [20]. For *S. reilianum*, studies have described that the sexual complementation of this fungus requires the production of pheromones [21]. In this case, psrDAPi is found in association with the soluble (psrDAPs) and membrane (psrDAPm) fractions. It is likely that the latter is an activity with functions similar to those of the membrane dipeptidyl aminopeptidases of *S. cereviseae*. This basidiomycete's genome has been shown to contain 5 genes that codify for possible dipeptidyl aminopeptidases; one of which is located in the membrane [16].

S. reilianum also produces of psrCPi activity, which is located in both the soluble and membrane fractions. The first is considerably inhibited by PMSF (serine protease inhibitor) and by metalloprotease inhibitors, which may indicate a mixture of enzymes that share the same catalytic activity. For the yeast *S. cerevisiae*, studies have described two carboxypeptidases of vacuolar location, denominated yscY and yscS. The first is a serine protease, the second, a zinc-dependent metalloprotease [5]. It has been shown that this yeast produces a polypeptide of cytoplasmic location called Ic, which is a specific inhibitor of the carboxypeptidase yscY. Determining its activity in cellular lysates requires adding sodium deoxycholate or previous incubation at acid pH to effectuate the dissociation of the endogenous inhibitor and the protease [22].

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This is also true for *S. reilianum*, because detecting psrCPi activity required adding sodium deoxycholate to the reaction mixture. This suggests the existence of an endogenous inhibitor that regulates this enzyme's activity.
The genome of this fungus contains 10 genes that codify for possible carboxypeptidases, six of thempossibly with vacuolar location [16].

Our study also found an additional activity of membrane-located psrCPi in *S. reilianum* that, in contrast to soluble psrCP, saw its activity reduced by PMSF and metalloprotease inhibitors, as well as by E-64 (cysteine protease inhibitor).

Meanwhile, descriptions of *S. cereviseae* mention a carboxypeptidase located in the membrane, denominated yscα, which requires both a serine and a cysteine group at the active site in order to perform catalysis [5]. In contrast to *S. reilianum*, carboxypeptidase activity at the biochemical level has not been found in *U. maydis* [10].

The present study found that *S. reilianum* has intracellular acid protease activity (psrPAi) that is likely similar to that reported for *U. maydis* (pumAi) [10]. Like psrPAi, production of the intracellular acid protease pumAi occurred in all culture media evaluated, and with every nitrogen source (peptone, ammonium, corn infusion, proline); however, in the case of proline it was associated with a logarithmic phase of growth [10].

Also, while in *U. maydis* the greatest specific activity of pumAi was obtained in a medium with proline [10], in that same medium, psrAi presented its lowest levels of activity. aspartyl protease, since it was inhibited by pepstatin A. The same results were found for pumAi from *U. maydis* [10]. Soluble intracellular aspartyl proteases have also been reported in such fungi as *S. cerevisiae*, *Aspergillus niger*, *Neurospora crassa*, *Coccidiodes immitis* and *Candida albicans* [23-27].

Inhibitor	Concen_ tration	Percent activity (%) psrDAPi Soluble fraction	psrDAPi Membrane fraction	psrCPi Soluble fraction	psrCPi Membrane fraction	psrAi Soluble fraction	psrAi Membrane fraction
	Without inhibitor	100 ± 3.2	100 ± 0.7	100 ± 0.4	100 ± 0.7	100 ± 1.7	100 ± 0.9
Bestatin	100 mM	100 ± 0.4	100 ± 0.6	100 ± 3.5	100 ± 1.6	100 ± 0.9	100 ± 0.1
	200 mM	100 ± 0.7	100 ± 0.1	100 ± 7.1	100 ± 3.1	100 ± 1.8	100 ± 0.3
Pepstatin A	5 µM	100 ± 0.1	100 ± 0.8	72 ± 0.5	83 ± .3	0 ± 0	0 ± 0
	25 µM	100 ± 0.5	100 ± 0.4	65 ± 2.9	46 ± 0.1	0 ± 0	0 ± 0
PMSF	1 mM	100 ± 0.1	100 ± 0.6	28 ± 3.2	87 ± 0.3	56 ± 4.9	0 ± 0
	5 mM	100 ± 0.1	76 ± 3.1	14 ± 1.1	4 ± 0.4	6 ± 0.3	0 ± 0
EDTA	1 mM	100 ± 0.1	100 ± 0.3	100 ± 0.1	81 ± 0.3	85 ± 0.4	21 ± 0.7
	10 mM	91 ± 0.1	100 ± 3.2	100 ± 0.5	19 ± 1.1	79 ± 2.4	20 ± 4.7
1-10-phenantroline	1 mM	100 ± 0.1	100 ± 0.1	100 ± 0.9	90 ± 1.4	ND	ND
	10 mM	75 ± 1.4	100 ± 7.3	100 ± 2.4	83 ± 0.55	ND	ND
E-64	10 µM	100 ± 0.9	100 ± 0.9	100 ± 0.1	72 ± 0.4	100 ± 0.9	28 ± 1.6
	50 µM	8 ± 3.2	100 ± 3.2	100 ± 3.5	58 ± 0.1	100 ± 0.9	27 ± 0.8

Table 2 Effect of proteolytic inhibitors on *S. reilianum* intracellular proteases

The activity of psrPAi turned out to be a soluble In the case of *S. cerevisiae*, it knows that the soluble aspartyl protease yscA is found in association with the vacuole [5], an organelle that presents acid pH values and is the site of important cellular functions, including autophagia [28, 29]. The fact that psrPAi is active at acid pH values strongly suggests that it is of vacuolar location. In *U. maydis*, the gene pep4 codifies for the vacuolar acid proteinase PrA, which plays an important role in that fungus' morphogenesis and virulence [11]. It is probable that psrPAi performs the same function in S. *reilianum.*

Upon performing cellular fractioning, our study found acid protease activity in the membrane and in the soluble fraction. Both were inhibited by pepstatin A, however, there is a difference in sensitivity to other inhibitors, since psrPAi of membrane is totally inhibited by E-64 and strongly inhibited by PMSF and EDTA, while soluble psrPAi is not inhibited by E-64 and only weakly inhibited by PMSF and EDTA. These findings could indicate that they are distinct enzymes that share the same catalytic activity, but are located in different sites in the cell. No pumAi activity has been reported in the membrane fraction of *U. maydis* [10]; an important difference with respect to *S. reilianum*.

The database for the genome of *S. reilianum* reports 8 genes that codify for possible aspartyl proteases [16]. One of these has already been associated with the aspartyl protease Eap1, codified by the gene with access key sr11394 [30], but the location of the others remains unknown. During the present study, we found that the activity of psrCP showed a significant decrease upon adding pepstatin A (an aspartyl protease inhibitor).

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Studies of the yeast *S. cerevisea* have reported that the vacuolar proteinase yscA activates other vacuolar proteases [5], so it may be that adding pepstatin A to the reaction mixture inhibits psrPAi affecting the activity of the protease psrCPi.

Conclusion

The results presented constitute the first report on the intracellular proteases psrDAPi, psrCPi and psrPAi of *S. reilianum*. It is probable that these enzymes have important functions during the life cycle and/or pathogenesis of this basidiomycete.

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