Characterization of the proteinases secreted by Sarocladium oryzae

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ABSTRACT

A biochemical survey was carried out to characterise the proteinase activity of proteases from the rice pathogenic fungus *Sarocladium oryzae* secreted into the culture medium. Azocasein was used as a substrate for total proteolytic activity assay and the maximum azocaseinolytic activity was obtained at pH 9.0 and using the synthetic substrate L-BAPA. These results and those obtained with specific inhibitors of the different classes of proteinases and with DTT as the activator of cysteine proteinases demonstrated that serine proteinases are the predominant proteases secreted *in vitro* by this fungus.

Keywords: Sarocladium oryzae, rice, proteinase, proteinase inhibitors

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RESUMEN

Caracterización de las proteinasas secretadas por *Sarocladium oryzae*. El examen bioquímico de las proteasas secretadas al medio de cultivo por el hongo patógeno del arroz *Sarocladium oryzae* permitió la caracterización de su actividad proteinasa. Para el ensayo de la actividad proteolítica total se usó como sustrato la azocaseina. A pH 9,0 se obtuvo la actividad azocaseinolítica máxima y utilizando el sustrato sintético L-BAPA. Estos resultados conjuntamente con los obtenidos con los inhibidores específicos de las diferentes clases de preoteinasas y con el DTT como activador de proteinasas del tipo cisteina, demostraron que entre las proteinasas secretadas *in vitro* por este hongo predominan las proteinasas del tipo serina.

Palabras claves: Sarocladium oryzae, arroz, proteinasa, inhibidores de proteinasas

*I*ntroduction

Rice sheath rot disease caused by the fungus *Sarocladium oryzae*, usually occurs on the flag leaf sheath (boot) that encloses the panicle in the rice plant. Panicles of sheaths affected before the emergence of the rot, turn brown or reddish brown and fail to produce grains. This disease affects yields in about 3-30%, sometimes reaching 85% [1].

Virulent strains of fungi secrete proteinases at significantly higher levels compared to less virulent strains. This proteinase activity causes plant cell death. It is also noteworthy that proteinase inhibitors are widespread in the plant kingdom. The possible roles of fungal proteinases in plant pathogenicity have been studied in only a few systems. Based on the fact that the structural component of the plant cell wall are glucids and proteins, proteolytic enzymes may play an important role in the pathogenesis of the wall, providing nitrogenous compounds to the fungus during infection [2].

Endopeptidases or proteinases cleave internal peptide bonds and are conveniently classified according to four mechanistic classes: serine proteinases, cysteine proteinases, aspartyl proteinases and metallo proteinases. In practice, the mechanistic class to which a proteinase belongs is first inferred from its in vitro characteristics, including its sensitivity to various inhibitors; its ability to hydrolyze specific proteins or peptides; its similarity to well-characterized proteinases and the pH range over which it is maximally active: serine proteinases (pH optimum 7.0-9.0), cysteine proteinases (pH optimum 4.0-7.0), aspartyl proteinases (pH optimum below 5.0), metallo proteinases (pH optimum 7.0-9.0) [3, 4, 5, 6, 7].

The characterization of *S. oryzae* extracellular proteinases *in vitro* was studied here. This information on fungi secreted proteinases could be useful to understand fungi growth and the infection process in which these enzymes are involved and may also provide the information needed to engineer the recombinant expression of specific inhibitors in rice to produce resistance against fungal infection.

*M*aterial and Methods

Strain

The *Sarocladium oryzae* strain was supplied by the Provincial Laboratory for Plant Health, Sancti Spíritus, Cuba.

Growth conditions and sample processing.

Standardized mycelial inocula (1 g wet weight per 100 mL) from 24 h Nutrient broth cultures were incubated while shaking (250 r.p.m.) at 28 ºC for 7 days in the Nutrient broth. The supernatant was harvested by culture vacuum filtration with a filter paper. It was concentrated to approximately 1/10 of the original volume by ultrafiltration (molecular mass cut-off 10000 Da). Protein concentration in the extract was assayed by the Bradford [8] method and using bovine serum albumin as a protein standard.

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Proteinase and proteinase inhibitor assays.

The proteinase assay procedure was adapted form Michaud *et al*. [9]. The following reagents were mixed to a final volume of 150 µL: 100µl of the appropriate reaction buffer, 20 µL of azocaseine (20 mg/mL in NaCl 0.15 M) and 30 µL of the sample (20-25 µg of total proteins).

Reaction buffers were: 0.1M citric acid-NaOH (pH 3.0- 6.0); 0.1M phosphate (pH 6.0-7.0) 0.1M Tris (pH 6.5-9.0); 0.1M Glycine-NaOH (pH 9.0-10.0) and 0.1M Na_2HPO_4-NaOH (pH 10.0-12.0). All buffers were adjusted with NaCl to the conductivity value of NaCl 0.15M at 20 ºC.

The reaction mixture was incubated for 24h at 37 ºC and stopped with 8 µL of 95% trichloroacetic acid (TCA) and residual azocaseine was removed by centrifugation for 20 minutes at 15 000 x g. Soluble peptides were measured using 70 µL of the supernatant at 405 nm in a Multiskan microplate reader. The blanks consisted of a complete reaction mixture where the enzyme extract was added after TCA.

Papain-like and trypsin-like activity of the sample was determined using Na-Benzoil-L-Arginine-*p*-nitroanilide (L-BAPA) as a substrate at 2.3 mM according to Houseman [10] the reaction was stopped with acetic acid to a final concentration of 15% (v/v).

One unit of proteinase activity was defined as the amount of enzyme required to produce a 0.01-absorbance unit at 405 nm for both substrates under reaction conditions.

For inhibition assays, proteinase samples were incubated for 1h in the presence of the following specific inhibitors: aprotinin, soybean trypsin inhibitor (STI) and phenylmethyl sulphonyl fluoride (PMSF) as the serine proteinase inhibitors; *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64), oryzacystatin I (OCI), oryzacystatin II (OCII), corn cystatin II (CCII), human stefin A (HSA), *p*-Cloro mercury-benzoic (*p*CMB), iodoacetamide (IAA) and cystatin as the cysteine proteinase inhibitors; pepstatin-A as aspartyl proteinase inhibitor; and ethylene diamine tetracetic acid (EDTA) as the metallo proteinase inhibitor. Antipain and leupeptin are serine as well as cysteine proteinase inhibitors. The cysteine proteinase activator dithiothreitol (DTT) was also tested. The inhibitors were used to classify the proteinase according to the effective concentrations recommended by Beynon and Salvesen [11] and Storey and Wagner [12].

Proteinase inhibitors and the activator were preincubated at 37 ºC, with the culture samples for 15 minutes, prior the addition of the substrate. All compounds were added in 20 µL of 0.15 M NaCl, except PMSF, E64, *p*CMB, IAA and pepstatin-A that were added in 20 µL of dimethyl sulphoxide (DMSO).

Results and discussion

The specific activity of the proteinases secreted by *S. oryzae* to the culture media was analysed over a wide range of pH (3.0 to 12.0), using azocaseine as a substrate for general proteinase activity (Figure 1). The maximum activity was obtained at pH 9.0, suggesting the predominance in the sample of serine and/or metallo proteinases [13, 14].

The absence of a defined peak suggests the presence of multiple classes of proteases, but we must bear in mind, when using proteins as substrates, that there are ionizable groups that might affect the substrate susceptibility producing changes in pH.

The L-BAPA is a specific synthetic substrate of cysteine proteinases: cathepsin B, cathepsin H and papain-like substances that work properly at acid pH. At a basic pH this compound is a serine proteinase substrate such as trypsin [15]. Figure 2 shows the behaviour of proteinases secreted by *S. oryzae* using L-BAPA as the substrate at different pH values (2.0-11.0). This graph also shows the papain and trypsin activity variation with the pH, using these as models of cysteine proteinase and serine proteinase respectively. The *S. oryzae* proteinase behaviour was trypsin-like, with a maximum activity at pH 9.0 such as the results obtained when using azocasein as the substrate.

It is known that antioxidant agents such as DTT, b mercaptoethanol and L-cysteine raise the cysteine proteinase activity at pH 4.0-7.0. Between pH 5.0 and 7.0 a very low enhancement of L-BAPA proteolysis was observed when the culture supernatant was incubated with DTT (Table 1). A similar result was obtained at pH 7.0 in the azocaseinolytic activity (1.4-fold). Nevertheless, in phytopathogenic insect gut fluids where the predominant proteolytic activity is cysteine-like, an activity enhancement of about 2-7 fold [16] has been observed in the presence of thiols compared to the control. In purified cysteine proteinases the activation by thiols can be higher; such as in the case of the papain activity at pH 5.0 using L-BAPA which increased the activity in our assay condi-

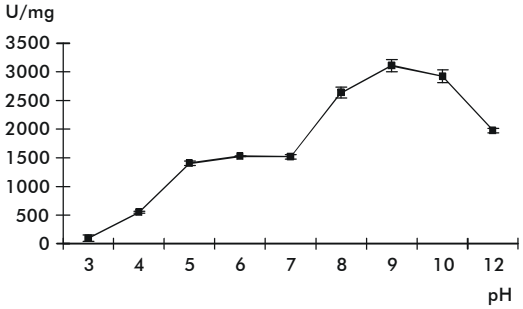

Figure 1. The effect of pH on the azocaseinolytic activity of proteinases secreted by *Sarocladium oryzae*. Data are the means of three measurements with the standard deviation.

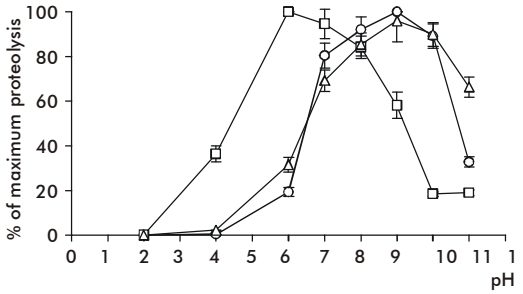

Figure 2. *S. oryzae* secreted proteinases behaviour with L-BAPA as the substrate at different pH values. Papain activity was tested in the presence of 2.5mM DTT. Data are the means of three measurements with the standard deviation. The curves represent Trypsin(O), Papain (□) and Sample (△).

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Table 1. Effect of DTT on the activity of proteinases secreted by *Sarocladium oryzae* using L-BAPA as the substrate.

	Specific Activity (U/mg)		
pH	Control	DTT	R
5.0	2632 ± 132[a]	3609 ± 180[b]	1.4
6.0	19248 ± 962[a]	17669 ± 883[a]	0.9
7.0	24812 ± 1241[a]	26917 ± 1340[a]	1.1
9.0	29692 ± 1346[a]	28077 ± 1403[a]	0.9

The control used distilled water instead of the DTT. The data are means of three measurements with their standard deviation. Means with different letters are significantly different from the control. A two tailed *t* test for unpaired data was used to evaluate differences in the DTT effect (P≤0.05). R is the ratio between the trial with DTT and that without DTT (Control).

tions up to160-fold with DTT 2.5 mM (Figure 2) These data indicate a low level of the cysteine proteinases among the proteinases secreted by *S. oryzae.* At pH 9.0 (the optimum pH for both substrates) no significant enhancement was observed in the activity of the sample tested with DTT. Although high activation is not expected for this pH value, other authors have found a residual activity and an activation of these enzymes with DTT at pH 9.0, using an azoalbumine substrate in insect gut fluid where the cysteine proteinases are predominant [16].

To determine the effect of specific inhibitors on the azocaseinolytic activity, the culture supernatant sample was incubated with the appropriate concentration of proteinase inhibitors that are specific for different classes of proteinases, at pH values of 9.0 and 7.0 since the pH of the assay mixture can greatly influence inhibitor-enzyme binding and therefore inhibitory activity. A pH of 7.0 is within the pH range of activity of the cysteine, serine and metallo proteinases and near the maximum value (pH 6.0) in the range reported for aspartyl proteinases. The percentage of inhibition in azocasein digestion provided evidence of the relative contribution of the inhibited classes of proteinases to the total fungus proteolytic activity.

The effect of specific proteinase inhibitors on the *S. oryzae* samples is presented in Table 2.

The serine proteinase inhibitors: PMSF, aprotinin, STI and the serine/cysteine proteinase inhibitors: antipain and leupeptin were the most effective in both pH values. In the case of antipain at pH 9.0 it was an activator instead of an inhibitor. The above mentioned were followed by the cysteine proteinase inhibitors: OCI, HSA, cystatin and E64 when assayed at pH 7.0, but at pH 9.0, except for E64 that maintains its activity, the others reduced their inhibitory effect or showed an activator behaviour. The inhibition observed after incubation with aspartyl and metallo proteinase inhibitors: both pepstatin A and EDTA, suggest the presence of this type of proteinase in the fungi culture supernatant although at low levels.

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Table 2. The effect of proteinase inhibitors on azocasein in *S. oryzae* supernatant samples.

Proteinase Inhibitor	Proteinase Specificity	Concentration (mM)	% Inhibition ± SD[a]	
			pH 7.0	pH 9.0
PMSF	Serine	3	56.0 ± 1.9	39.0 ± 1.2
Antipain	Serine / Cysteine	1	44.5 ± 2.6	-12.5 ± 0.8
Leupeptin	Serine / Cysteine	0.01	38.1 ± 1.1	20.2 ± 1.0
Aprotinin	Serine	0.05	27.7 ± 1.5	25.8 ± 1.2
OCI	Cysteine	0.01	19.1 ± 1.0	-0.1 ± 0.1
STI	Serine	0.01	17.0 ± 1.2	27.0 ± 1.4
E64	Cysteine	0.01	9.2 ± 0.6	9.3 ± 0.3
Cystatin	Cysteine	5	9.0 ± 1.6	-3.8 ± 0.5
HSA	Cysteine	0.01	15.0 ± 1.5	2.9 ± 0.1
OCII	Cysteine	0.01	6.5 ± 0.4	0.0 ± 0.1
Pepstatin-A	Aspartyl	0.01	4.5 ± 0.6	1.9 ± 0.2
EDTA	Metallo	1	2.8 ± 0.1	3.5 ± 0.5
IAA	Cysteine	1	2.4 ± 1.4	-10.7 ± 1.6
CCII	Cysteine	0.01	0.2 ± 0.1	0.0 ± 0.2
*p*CMB	Cysteine	2	-1.4 ± 1.7	-10.1±1.0

[a]Values are the means ± standard deviation of three different measurements of the sample with an inhibitor versus its control without it. The minus sign refers to activation.

In general a reduction was observed in the inhibitory activity of cysteine and aspartyl proteinase inhibitors when they were tested at pH 9.0 compared to the values obtained at pH 7.0. This result could be explained because the pH at which the assay was conducted was far from the pH at which the inhibitor would be expected to be active. However, except for STI, that was more effective at pH 9.0, the serine proteinase inhibitors showed an unexpected lower activity at pH 9.0.

As a whole, the results indicate that serine proteinase is the predominant type of proteinase secreted into the culture medium by *Sarocladium oryzae* during its growth in our experimental conditions.

The presence of serine proteinase in a pathogenic fungus was also observed by Dobinson *et al*. [17] in *Verticillium dahliae.* Other plant pathogenic fungi such as *Scletotina sclerotium* a widespread necrotrophic fungus, has several acid proteinases including a non-aspartyl proteinase that is secreted during growth on plant cell walls [1]. Nevertheless a variety of types of proteinases have been isolated from fungi that interact with plants [2, 18].

Proteinases in pathogenic fungi might contribute to the virulence and provide a nitrogen supply for the organism when inorganic nitrogen in the environment has been depleted [18, 19, 20]. According to this role, this study is a contribution to fungus biochemistry and physiology and also offers information for the expression of different serine proteinase inhibitors as a promising strategy to engineer rice plants with enhanced resistance to *Sarocladium oryzae* and allows to explore novel candidate molecules with a potential in molecular breeding strategies.

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