Characterization of the proteases secreted by the rice pathogenic fungus Pyricularia grisea

Yeleiny Machin, ✉ Carlos Hernández, Duniesky Martínez, Julio Alfonso

División de Plantas y Enzimas, Centro de Ingeniería Genética y Biotecnología, CIGB
Apartado 83, CP 60200, Sancti Spíritus, Cuba
E-mail carlos.hernandez@cigb.edu.cu

RESEARCH

ABSTRACT

Culture supernatants from the rice pathogenic fungus *Pyricularia grisea* were characterized by general proteolytic assays. Protease activity, as measured by azocasein hydrolysis, peaked over a pH range of 7.0 - 9.0 with an optimum at pH 8.0. This, together with the results from zymograms of gelatin-containing SDS-PAGE gels combine with specific protease inhibitors, suggest the presence of serine-like proteases and metalloproteases. The ability of the secreted proteases to hydrolyze specific synthetic substrates, the determination of their optimal pH for hydrolysis, and their sensitivity to protease inhibitors confirmed the presence of elastase-, leucine aminopeptidase-, arginine aminopeptidase-, carboxypeptidase A- and B-like activities in culture supernatants from *P. grisea*. This study is a contribution to the knowledge of the biochemistry and physiology of this fungus, and also offers information for the expression of protease inhibitors as a promising strategy for engineering rice plants with enhanced resistance to *Pyricularia grisea*.

Keywords: *Pyricularia grisea*, rice, protease, protease inhibitors

Biotecnología Aplicada 2008;25:111-116

RESUMEN

Caracterización de las proteasas secretadas por el hongo patógeno del arroz *Pyricularia grisea*. Se ensayó la actividad de las proteasas secretadas al medio de cultivo por el hongo patógeno del arroz *Pyricularia grisea*. La actividad proteolítica general se midió utilizando como sustrato azocaseína, la mayor actividad se observó en un rango de pH de 7.0 a 9.0 con una actividad óptima a pH 8.0. Esto sugiere, junto a los zimogramas de geles de SDS-PAGE con gelatina en combinación con inhibidores específicos de proteasas, la presencia de proteasas del tipo serino y metaloproteasas. La capacidad de las proteasas secretadas para hidrolizar sustratos sintéticos específicos, la determinación del pH al cual ocurre la máxima actividad, y su sensibilidad a inhibidores de proteasas específicos, confirmaron la presencia de actividades similares a elastasa, leucina-aminopeptidasa, arginina-aminopeptidasa, carboxipeptidasa A y carboxipeptidasa B en las secreciones de *P. grisea*. Este estudio es una contribución al conocimiento de la bioquímica y fisiología de este hongo y ofrece información para la expresión de inhibidores de proteasas, como una estrategia prometedora en la transformación de plantas de arroz para la resistencia al hongo *Pyricularia grisea*.

Palabras clave: *Pyricularia grisea*, arroz, proteasas, inhibidores de proteasas

*I*ntroduction

Rice blast is caused by the fungus *Pyricularia grisea*, also known as *Pyricularia oryzae*. The first symptoms of the disease are the presence of elliptical gray-white lesions with reddish edges on the leaves (leaf blast) and stems of the plant, running parallel to the long axis of the affected organ. Greater damage occurs when the fungus spreads to the area below the seed head of the plant, causing it to break off (rotten neck); additionally, rice blast may also prevent the maturation of the rice grains (panicle blast). Other possible manifestations of the disease are the presence of dark-brownish, blackish lesions at the stem nodes, which may cause stem breakage and ultimately result in the death of the stem or panicle. In all, the most commonly observed symptom of blast occurs when the fungus attacks the base or neck of the panicle (frequently referred to as "rotten-neck", as noted above). The rotten infection site will frequently break [1].

Rice blast occurs in most of the rice producing areas of the world. It is considered one of the most important diseases of this crop due to its wide distribution and destructiveness, causing severe yield losses under the appropriate environmental conditions [2, 3].

The presence of extracellular proteases have been reported in a number of fungal plant pathogens, and it has become increasingly clear that proteases produced by pathogenic fungi play an important role in host tissue invasion, providing nitrogenous compounds to the fungus during infection [4-7]. Engineering the recombinant expression of specific protease inhibitors in rice is a promising strategy to produce resistance against fungal infection. However, because of the variability in proteases between fungi [7-9], and the limited spectrum of activity of these inhibitors [10], the expression in plants of a particular protease inhibitor may not result in a broad pattern of resistance. The inhibitor must therefore be previously matched for its expression against the secreted proteases of the target pathogen, which requires the knowledge of the proteases secreted by the fungus and the way they interact with the available inhibitors.

The aim of this study is to characterize the proteases secreted by *Pyricularia grisea* according to the range of pH at which they show maximum activity; their sensitivity to various inhibitors and their ability to hydrolyze specific proteins and peptides.

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✉ Corresponding author

Materials and methods

Chemicals and Equipment

All substrates, protease inhibitors and electrophoretic reagents were purchased from Sigma Chemical Co., (St. Louis, MO). Spectrophotometric measurements were made using a Shimadzu Spectrophotometer UV-1203 and the photometric measurements were made using a SUMA PR-251 microplate reader.

Strain

The *Pyricularia grisea* strain was supplied by Engineer Jorge Luis Fuentes from CEADEN, Havana, Cuba.

Growth conditions and sample processing

Pyricularia grisea was kept on potato-dextrose agar at 30 ºC, and inocula were prepared from 7-day-old cultures by flooding with 10 mL of sterile distilled wa-ter and scraping off the agar plates. Liquid cultures were grown by shaking (100 rpm.) at 28 °C, for 6 days in 100 mL of a minimal liquid medium containing K_2HPO_4 0.1% (w/v), $MgSO_4$ x $7H_2O$ 0.05% (w/v), KCl 0.05% (w/v), $FeSO_4$ x $7H_2O$ 0.001% (w/v), glucose 1% (w/v) and supplemented with rice extract (0.1% proteins (w/v)). The medium was sterilized by filtration (0.2 µm), and the proteases from the rice leaf extract in the medium were inactivated by incubation for 48 h at 60 ºC and two hours in an ultrasonic bath. Culture supernatants were harvested by vacuum filtration with filter paper, and the mycelia were dried at 95 ºC for 12 h and the weighed. The total protein concentration in leaf extracts was assayed by the Bradford method [11], using bovine serum albumin as a standard.

For the kinetic study of enzyme production, triplicate cultures were carried out in 100 mL of the culture medium, each containing 10 mL of inocula, in the culture conditions outlined above. Samples were taken at different time periods ranging from 24 h to 7 days.

Enzyme assays

All assays were carried out in triplicate and blanks were used to account for the spontaneous breakdown of substrates and sample effects. Reaction buffers were: 0.1 M citric acid-NaOH (pH 2.0-3.0), 0.05 M citrate-citric acid (pH 3.0-6.0); 0.1 M Tris (pH 7.0-9.0); 0.1 M Glycine-NaOH (pH 9.0-10.0); 0.05 M sodium borate-NaOH (pH 9.0-10.0) and 0.1 M Na_2HPO_4-NaOH (pH 10.0-12.0). All buffers were adjusted with NaCl to the conductivity value of 0.15 M NaCl at 20 ºC.

Unless otherwise stated, all protease activity assays were adapted from Ortego *et al* [12]. The following reagents were mixed to a final volume of 167 µL: 117-137 µL of the appropriate reaction buffer, 10 µL of the substrate and 20-40 µL of the sample. In all cases the reaction mixture was incubated at 30 ºC.

Non-specific protease activity was determined using a 20 mg/mL sulphanilamide-azocasein solution; the reaction was incubated for 24 h and then stopped by the addition of 9.28 µL of 95% (w/v) ice-cold trichloroacetic acid. The solution was centrifuged at 15 000 *g* for 20 min and the absorbance of the supernatant measured at 405 nm. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance change of 1.000 at 405 nm after 24 h of incubation at 30 ºC.

The assays for hydrolytic activities against specific synthetic substrates were conducted as follows: Chymotrypsin-, cathepsin G- and elastase- like activities were assayed with SA_2PPpNA (N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide), incubating the reaction mixtures for 6 h. Elastase-like activities were also assayed with SA_2PLpNA (N-succinyl-alanine-alanine-proline-leucine-p-itroanilide), but using an incubation time of 3 h. Trypsin-, papain, cathepsin B- and cathepsin H-like activities were assayed using BApNA (Na-benzoyl-L-Arginine-p-nitroanilide), whereas papain- and cathepsin L-like activities were detected with ZPApNA (carbobenzoxy-phenyl-alanine-Arginine-p-nitroanilide), incubating the reaction for 24 h for both substrates. Cysteine proteases were measured in presence of 2.5 mM DTT. The measurements of leucine amino-peptidase- and arginine aminopeptidase-like activities were done by incubation for 2 h with Leu-pNA (leucine-p-nitroanilide) and for 6 h with Arg-pNA (arginine-p-nitroanilide), respectively.

All substrates containing p-nitroanilide were dissolved in 99% DMSO, and were used at a final concentration of 1 mM, stopping the reactions in all cases by adding 18.62 µL of 99.7% acetic acid. After incubation, the reactions were centrifuged at 15 000 *g* for 5 min and the absorbance of the supernatants was measured at 405 nm. The rate of the reaction was calculated from the amount of p-nitroanilide (pNA) released using an absorptivity of 10.2 $cm^2/\mu mol$ calculated under our conditions.

Carboxypeptidase A-like and carboxypeptidase B-like activities were assayed with 1 mM HPA (hippuryl-phenylalanine) and 1 mM HA (hippuryl-L-arginine), respectively. HPA was added in DMSO 99% (w/w) and HA was added in 0.15 M NaCl. The reactions were incubated for 4 h and stopped with acetic acid as described above. The rate of the reaction was calculated by estimating the amount of released amino acid using the ninhydrin procedure [13], and the reaction buffer was 0.05 M borate-NaOH (pH 9.0-10.0), since a glycine-based buffer would react with ninhydrin. The units of enzymatic activity (U) are expressed in nmol of phenylalanine released/minute and nmol of arginine released/minute (for carboxy-peptidase A and carboxypeptidase B, respectively),

The aspartyl protease activity was measured in 1 mL of the reaction buffer containing 20 µL of the sample and 2% (w/v) hemoglobin (added in 100 µL 0.15 M NaCl) [14]. The reaction was incubated for 24 h and stopped by adding 56 µL of 95% (w/v) ice-cold trichloroacetic acid. The solution was then centrifuged at 15 000 *g* for 20 min, and the supernatant was monitored by a spectrophotometer at 280 nm.

Chymotrypsin-, cathepsin G- and subtilisin-like activities were measured using Ac-phe-β-NE (acetyl-phenylalanine-β-naphthyl-ester). The procedure was adapted from Barrett [15]. The activity was measured in 500 µL of the reaction buffer containing 20 µL of sample and 15 µL of the substrate at a final concentration of 1 mM. The reaction was incubated for 24 h and stopped by adding $HgCl_2$ to a final con-

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centration of 10 mM, followed by incubation at room temperature for 10 min. Afterwards, 500 µL of 0.2 mg/mL of Fast-Garnet were added and incubated for an additional 30 min, reading the results by a spectrophotometer at 520 nm.

Effects of protease inhibitors and activators *in vitro*

The proteolytic activities secreted by *P. grisea* were assayed in the presence of the following specific inhibitors: serine protease inhibitors STI (soybean trypsin inhibitor) and PMSF (phenylmethyl-sulfonyl fluoride), serine and cysteine protease inhibitors TPCK (N-tosyl-L-phenylalanine chloromethyl ketone) and TLCK (Na-p-tosyl-L-lysine chloromethyl ketone), cysteine protease inhibitors E-64 (L-trans-epoxy-succinyl-leucylamido-(4-guanidino)-butane) and IAA (iodoacetamide), the aspartyl protease inhibitor pepstatin-A, the metalloprotease inhibitor EDTA (ethylenediamine tetraacetic acid), and several inhibitors and activators with variable effects among different protease types: $CuCl_2$, $CaCl_2$, $ZnSO_4$, $MgCl_2$, $MnCl_2$, $CoCl_2$ and DTT (dithiothreitol).

The protease inhibitors and activators were pre-incubated at 30 ºC with the samples of the culture supernatant for 15 min at the optimum pH for each proteolytic activity prior to adding the substrate. All compounds were dissolved in 0.15 M NaCl except for TPCK, E-64 and pepstatin-A, which were dissolved in DMSO 99% (w/w), and PMSF, which was dissolved in absolute ethanol. The inhibitors and activators were used for the determination of the catalytic type of the protease tested, following the effective concentrations recommended by Beynon and Salvesen [16].

Zymograms

The electrophoretic detection of proteolytic forms was performed using 0.1% (w/v) gelatin-containing 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gels according to Michaud *et al.* [17]. The samples were diluted two-fold in electrophoresis sample buffer 2X (125 mM Tris-HCl pH 6.8, SDS 4% (w/v), glycerol 20% (v/v), bromophenol blue 0.02% (w/v)) and subjected to electrophoresis using a Bio-Rad Mini-Protean II Electrophoresis Cell system. After electrophoresis at 4 ºC, 200 V, the gels were transferred to a 2.5% (v/v) solution of Triton X-100 and incubated for 30 min at room temperature to allow the renaturation of the proteinases, followed by incubation for 24 h at 37 °C in activation buffer (50 mM Tris-HCl pH 8.0). The proteolysis was stopped by transferring the gels to a staining solution composed of 0.1% (w/v) Coomassie Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining in 25% (v/v) methanol and 10% (v/v) acetic acid.

To inhibit serine-like proteinases, the sample of the supernatant was pre-incubated with 5.0 mM PMSF for 20 min at 30 ºC, while using a parallel negative control where the inhibitor solution was replaced by its corresponding solvent. The inhibition of metalloproteinase activity after electrophoresis was achieved by dividing the gel into two halves and incubating one of them in the activation buffer and the other in the same buffer supplemented with 8.0 mM EDTA, under the conditions shown above.

An azocollagenolytic activity assay was adapted from Michaud *et al.* [18] and used for the normalization of the amounts of enzyme loaded in the gelatin-containing electrophoretic experiments. Three milligrams of azocollagen were added to 960 µL of 0.1 M Tris buffer, pH 8.0 and 40 µL of the sample. The reaction was incubated for 3 h at 250 rpm, 37 ºC, and stopped by centrifugation at 15 000 *g* for 10 min. The results were determined by measuring the absorbance of the supernatant at 520 nm.

Statistical Analysis

The kinetics of protease synthesis was studied using a completely randomized analysis of variance. Multiple comparisons of the means were conducted using the *Student-Newman-Keuls test*, and the effect of specific protease inhibitors and activators on the hydrolysis of the protein and synthetic substrates was analyzed with an unpaired *t-test*. All statistical analyses were conducted with the COSTAT 2.04 (CoHort Software 1986) statistical software application on a personal computer.

Results and discussion

The time course of extracellular protease secretion into the medium was used to select the incubation time of the culture. Hydrolytic activity was measured every 24 h for 7 days, with azocasein as the general protease substrate and with the specific synthetic substrates SA_2PPpNA and Leu-pNA for serine-endopeptidase-like and leucine-aminopeptidase-like activities, since the latter are two of the most frequently reported proteases in fungal secretions [19].

Figure 1 shows that the azocaseinolytic activity values increased significantly with culture time, reaching a maximum at the 7th day. A similar behavior was

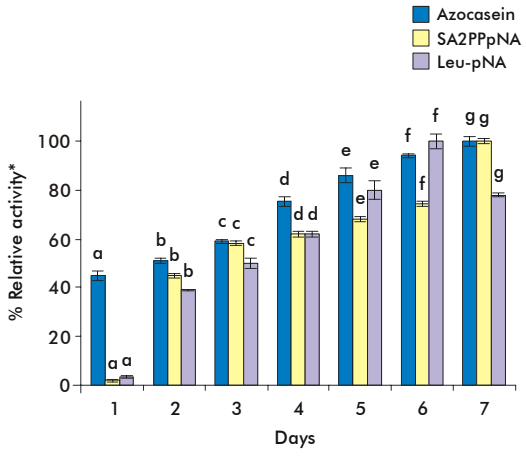

Figure 1. Time course of extracellular protease activity during the culture of *P. grisea*.
*Data are expressed in percentage, with 100 as the highest activity of the samples in each substrate at its optimal pH (pH 8.0 to azocasein and pH 9.0 to SA_2PPpNA and Leu-pNA). Data are the means of triplicate measurements with standard errors. Different letters show significant differences between days for each substrate (*Student-Newman-Keuls test*, $P \leq 0.05$).

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obtained using the SA_2PPpNA substrate. However, the maximum leucine aminopeptidase-like activity was reached at the 6th day of growth, followed by a sharp decrease at the 7th day to the levels present at the 5th day. These differences could be explained by a different association with growth kinetics for different proteases, and/or due to the induction of a decrease in the secretion of one or more proteases owing to the depletion of some substrates in the culture medium. For these reasons, all subsequent samples were taken at the 6th day for all the remaining experiments.

The presence of secreted proteases of different mechanistic classes appears to be widespread among the filamentous fungi. While serine-, aspartyl- and metalloproteases are common, there are only a few reports on the presence of cysteine proteases [19]. Additionally, recent reports have indicated the presence of aminopeptidase, carboxypeptidase and dipeptidyl-peptidase activities in pathogenic fungi [20].

The presence of elastase-, leucine aminopeptidase-, arginine aminopeptidase-, carboxypeptidase A- and B-like activities in the secretions of *P. grisea* was confirmed by the ability of the secreted proteases to hydrolyze specific synthetic substrates, their optimal pH for proteolysis, and their sensitivity to protease inhibitors. Quilis *et al.*, also found a carboxypeptidase B-like activity in mycelial extracts of this fungus, which was fully inhibited by the potato carboxypeptidase inhibitor [21].

The *P. grisea* genome contains a large, diverse set of proteins that can be secreted, including enzymes involved in the degradation of the plant cell wall [22]. Interestingly, the predicted secreted proteome of *P. grisea* includes a carboxypeptidase B homolog, in agreement with the carboxypeptidase B-like activity identified in this study.

The pH profiles for the proteolytic activity of the secretions of *P. grisea* are presented in Figure 2. When using azocasein as the protein substrate, the highest activities were found across a range of pH of 7.0 - 9.0, with an optimum at pH 8.0; suggesting the presence of serine-like proteases and/or metalloproteases [23-26]. These pH profiles agree with those obtained with specific substrates, since the assays using SA_2PppNA, SA_2PLpNA and Leu-pNA presented an optimum at pH 9.0, whereas optimal hydrolysis of Arg-pNA occurred at pH 8.0. The activity against the HPA and HA substrates peaked at pH 7.0 - 7.5 and 7.0, respectively.

Table 1 summarizes the hydrolytic activities of the extracellular proteases of *P. grisea* against all general and specific substrates analyzed. No proteolytic activity was detected against Ac-phe-β-NE (specific for Chymotrypsin-, cathepsin G- and subtilisin-like activities) after 24 h of incubation. However, there was detectable activity against SA_2PPpNA (specific for elastase-, chymotrypsin-, and cathepsin G-like activities). This suggests the presence of an elastase-like activity in the samples, and agrees with the results obtained from the assays with SA_2PLpNA. There were no cysteine-, aspartyl-, trypsin- or plasmin-like activities detected in the enzyme assays, in spite of previous reports on the presence of aspartyl-like activities in the pathogenic fungi *Sarocladium grisea*; *Botrytis cinerea* and *Sclerotinia sclerotiorum* [9, 27, 28].

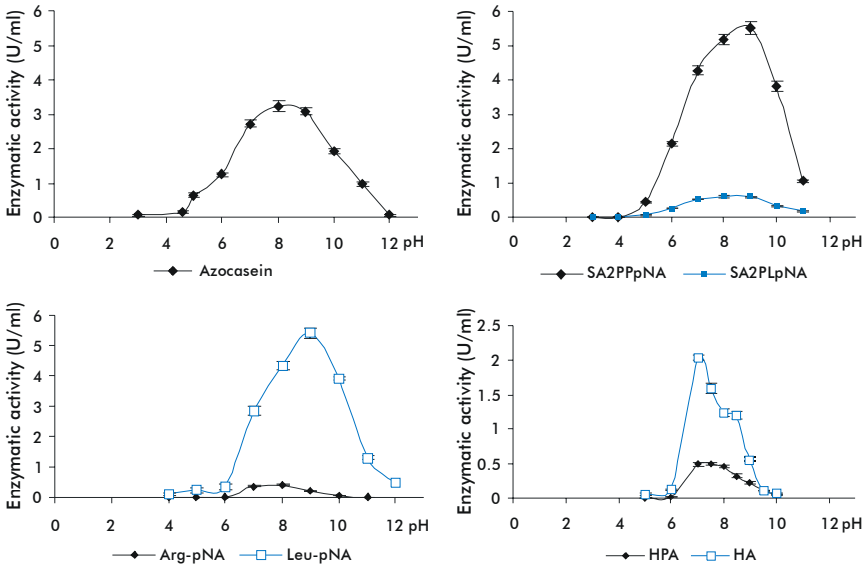

Figure 2. The effect of pH on the rate of hydrolysis of proteins and specific substrates by the secreted proteases of *P. oryzae*. For general proteolytic activity against azocasein, one unit (U) of protease activity is defined as the amount of enzyme required to produce an absorbance change of 1.000 at 405 nm in 24 hr of incubation at 30 °C. Units of enzymatic activity against specific substrates are expressed in nmol of pNA released/minute or in nmol of phenylalanine released/minute and nmol of arginine released/minute (for carboxypeptidase A and carboxypeptidase B, respectively). Data represent the mean of three measurements with standard errors.

Table 1. Summary of the hydrolytic activity of the extracellular proteases secreted by *P. grisea* against all general and specific substrates assayed

Protease type (specificity)	Substrate	Specific activity*
Proteases	Azocasein	1.06 ± 0.05
Serine-proteases		
(Chymotrypsin, Elastase and Cathepsin G)	SA2PPpNA	1.83 ± 0.06
(Elastase)	SA2PLpNA	0.20 ± 0.04
(Trypsin and Plasmin)	BApNA	No activity
(Chymotrypsin, Cathepsin G and Subtilisin)	Ac-phe-ß-NE	No activity
Cysteine-proteases		
(Papain, Cathepsin B and Cathepsin H)	BApNA	No activity
(Papain and Cathepsin L)	ZPApNA	No activity
Aspartyl proteases		
(Cathepsin D and Pepsin)	Hemoglobin	No activity
Aminopeptidases		
(Leucine-aminopeptidase)	Leu-pNA	1.80 ± 0.05
(Arginine-aminopeptidase and Cathepsin H)	Arg-pNA	0.13 ± 0.01
Carboxypeptidases		
(Carboxypeptidase A)	HPA	0.17 ± 0.01
(Carboxypeptidase B)	HA	0.67 ± 0.01

*Specific activity as nmol of substrate hydrolyzed/min/g of dry mycelium, except for azocaseinolytic activity as U/g of dry mycelium, where one unit of protease activity (U) is defined as the amount of enzyme required to produce an absorbance change of 1.000 at 405 nm in 24 hr of incubation at 30 °C. The activity of the samples in each substrate was measured at its optimal pH. Data represent the mean of three measurements with standard errors.
No activity means that there is no hydrolytic activity in the pH range of 2.0-10.0, except for aspartyl proteases assayed at pH 2.0 - 5.0.

The proteolytic activity of the secreted proteases from *P. grisea* was further characterized by examining their sensitivity to specific protease inhibitors (Table 2). The cysteine protease inhibitors: IAA and E64, the aspartyl protease inhibitor: pepstatin A, and the trypsin and other serine - and cysteine-proteinase inhibitor: TLCK had no inhibitory or activating effect against

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Table 2. Effect of protease inhibitors and activators on the hydrolysis of protein and synthetic substrates by the secreted proteases of *P. grisea* at their optimum pH

		% Residual activity*						
Inhibitor/Activator (conc.)	Specificity	Azocasein (pH 8.0)	SA2PPpNA (pH 9.0)	SA2PLpNA (pH 8.0)	Leu-pNA (pH 9.0)	Arg-pNA (pH 8.0)	HPA (pH 7.0)	HA (pH 7.0)
STI (13.8 μM)	Serine	73.2 ± 0.5	28.7 ± 1.2	40.7 ± 2.6	ne	ne	ne	ne
TLCK (1 mM)	Serine/Cysteine	ne	ne	ne	ne	ne	ne	ne
TPCK (1 mM)	Serine/Cysteine	77.0 ± 3.0	68.5 ± 4.9	62.3 ± 2.2	ne	ne	ne	ne
PMSF (2 mM)	Serine	78.0 ± 2.0	16.9 ± 4.0	23.5 ± 2.1	ne	ne	154 ± 11	ne
E-64 (10 μM)	Cysteine	ne	ne	ne	ne	ne	ne	ne
IAA (1 mM)	Cysteine	ne	ne	ne	ne	ne	ne	ne
Pepstatin A (1 μM)	Aspartyl	ne	ne	ne	ne	ne	ne	ne
EDTA (5 mM)	Metallo	60.0 ± 2.7	ne	ne	15.6 ± 2.1	5.7 ± 0.6	2.1 ± 0.1	45.4 ± 2.3
Cu2+ (1 mM)	V	68.7 ± 3.6	169 ± 4.0	123 ± 4.0	5.3 ± 0.3	ne	8.1 ± 0.4	1.1 ± 0.1
Ca2+ (1 mM)	V	120 ± 2.0	178 ± 10	142 ± 2.0	ne	138 ± 1.0	243 ± 12	ne
Zn2+ (1 mM)	V	49.2 ± 1.8	ne	ne	334 ± 9.0	65.7± 2.6	189 ± 13	ne
Mg2+ (1 mM)	V	122 ± 4.0	164 ± 2.0	ne	255 ± 9.0	165 ± 2.0	37.8 ± 2.6	45.8 ± 3.0
Mn2+ (1 mM)	V	122 ± 4.0	128 ± 6.0	145 ± 4.0	ne	12.7± 0.8	187 ± 11	30.4 ± 1.5
Co2+ (1 mM)	V	iq	199 ± 5.0	193 ± 6.0	461 ± 6.0	ne	492 ± 29	ne
DTT (5 mM)	V	ne	ne	ne	0	59.5 ± 1.3	0	44.7 ± 2.0

*Values are the means with standard errors of three determinations treated with an inhibitor or activator versus their corresponding controls without them. No effect (ne) was considered for data without significant differences compared to the control, by using a Studtent's-test ($P \geq 0.05$). V: variable effect according to the protease type. iq: the reagent chemically interferes with the assay.

the hydrolysis of any substrate. The azocaseinolytic activity was inhibited by the specific serine protease inhibitors STI, PMSF and TPCK. Azocasein hydrolysis was also inhibited by the metalloprotease inhibitor EDTA as well as by Cu^{2+} and by Zn^{2+}; it was activated, however, by Ca^{2+}, Mg^{2+} and Mn^{2+}.

The hydrolysis of SA_2PPpNA and SA_2PLpNA was inhibited by PMSF, STI and TPCK. Among the several serine proteinase inhibitors tested, PMSF was the most effective one. The selective effect of the serine proteinase inhibitors confirmed the predominant presence of an elastase-like activity among the serine proteinases in the culture medium. This activity was also activated by Co^{2+} and no effect was observed when adding EDTA, suggesting that this elastase is most likely not a metalloprotease. The hydrolysis of SA_2PPpNA and SA_2PLpNA was also activated by the metal ions Cu^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+} or Cu^{2+}, Ca^{2+} and Mn^{2+}, respectively.

The exopeptidase activity assayed with the specific substrates Leu-pNA, Arg-pNA, HPA and HA did not decrease in the presence of STI, TPCK and PMSF, except for the hydrolysis of HPA, which was unexpectedly activated by PMSF. The hydrolysis of Leu-pNA was inhibited by EDTA, DTT and Cu^{2+} and activated by the divalent metal ions Zn^{2+}, Mg^{2+} and Co^{2+}. Arg-pNA hydrolysis was inhibited by EDTA, DTT, Zn^{2+} and Mn^{2+}; and activated by Ca^{2+} and Mg^{2+}.

According to previous reports, divalent cations can have different effects on aminopeptidase activity. Zn^{2+} inhibits the arginine aminopeptidase of the bacterium *Lactobacillus sakei* [29] but activates the leucine aminopeptidase of the fungus *Aspergillus sojae* [30] and the bacterium *Aeromonas salmonicida* [31] Bolumar *et al*. [32] reported, in contract to our results, that an arginine aminopeptidase from the fungus *Debaryomyces hasenii* was activated by Mn^{2+} and inhibited by Mg^{2+}; and both ions have been reported as activators of the leucine aminopeptidase activity from Hog kidney [33]. Co^{2+} has also been described as an activator of arginine and leucine aminopeptidase activities by Chien *et al*. [30] and Bolumar *et al*. [32] respectively.

In the case of Cu^{2+} our findings agree with those reported for the inhibition of the leucine amino-peptidase activity from the mite *Tyrophagus putrescentiae* [34], from larvae of *Sesamia nonagrioides* [12] and from Colorado potato beetle larvae [14], even though in our conditions Cu^{2+} was not inhibitory for the arginine aminopeptidase-like protease. The latter disagrees with previous reports for arginine aminopeptidases from the bacterium *Lactobacillus sakei* [29] and the fungus *D. hasenii* [32].

The hydrolysis of the carboxypeptidase-A substrate HPA was inhibited by EDTA, DTT, Cu^{2+} and Mg^{2+}; and activated by Ca^{2+}, Zn^{2+} and Mn^{2+}. Additionally, the hydrolysis of HA was inhibited by EDTA, DTT, Cu^{2+}, Mn^{2+} and Mg^{2+}. The inhibitory activity exhibited by EDTA and DTT in the hydrolysis of all exopeptidase substrates used in this study indicates that either they are metalloproteases or they require divalent cations for their activity.

Gelatin-containing SDS-PAGE gels of secreted proteases under non-reducing conditions are shown in figure 3. Two bands with gelatinolytic activity were resolved from the extracellular proteases of *P. grisea*. The non-inoculated medium did not show any gelatinolytic activity; therefore the proteases detected come from fungal secretions. PMSF inhibited only the activity of the upper band (Figure 3A), indicating that this species is a serine-like protease, which may well correspond to the elastase-like activity identified in the *P. grisea* secretions. In order to identify the proteolytic activity of the lower band, the gel was divided into two halves after the end of the electrophoretic run, incubating one of them in the activity buffer as a control and the other in the same buffer but containing

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0.8 mM of EDTA (Figure 3B). In this case the lower band was totally inhibited by EDTA, indicating that it corresponds to a metalloprotease. The detection of only two proteases by this technique may be explained by the fact that not all enzymes are able to refold after electrophoretic migration [35]. Additionally, some of the proteases secreted by *P. grisea* may not hydrolyze gelatin; and since molecular weight is not the only factor influencing relative migration [17], the possibility that each band corresponds to more than one proteolytic species can not be excluded.

The presence of proteases with the same mechanistic class as those found in *P. grisea* has been reported for other fungi. Frosco *et al*. [36] purified an elastase in the culture medium of *Aspergillus fumigatus*. A leucine-aminopeptidase was purified to homogeneity from *A. sojae* [30], and the same activity has been detected in liquid and agar cultures of *Chondrostereum purpureum* [37]. Bolumar *et al*. [32] purified an arginine-aminopeptidase from *D. hansenii*. Metallocarboxy-peptidases have been found mainly in bacteria and animals; a carboxypeptidase A gene involved in the acquisition of nutrients from the host was identified in the fungus *Metarhizium anisoplace* [38]. Roncero *et al*. [7] have isolated a gene encoding an extracellular serine protease from *Fusarium oxysporum* f.sp. *lycopesici*, which has been hypothesized to play a significant role in the interaction of these fungi with their host, in the same way as the subtilisin-like serine proteases isolated from the entomopathogen *Metarhizium anisoplace* and the endophyte *A. typhinum* [8, 39].

The strong induction of different serine protease inhibitors in a blast-resistant rice mutant during blast infection suggests that these small proteins play important roles in blast resistance [40].

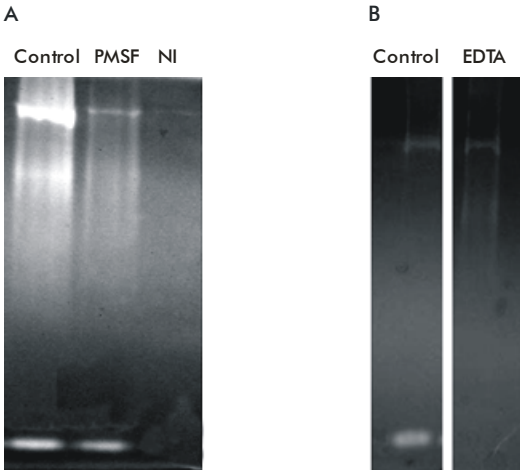

Figure 3. Zymogram showing gelatinolytic activity of the secreted proteinases by *P. grisea* treated with PMSF (A) and EDTA (B) and without them (Control). NI: non-inoculated medium. A total of 17 mU of azocollagenolytic activity were loaded per lane. One unit of azocollagenolytic activity is defined as the amount of enzyme required to produce an absorbance change of 1.000 at 520 nm in 3 hr of incubation at 37 °C and at pH 8.0.

This study is a contribution to fungal biochemistry and physiology and also offers information for the engineering of rice plants with enhanced resistance to *Pyricularia grisea* through the expression of protease inhibitors, which is emerging as a promising strategy for pest control. However, the presence of at least two mechanistic classes of proteases in this fungus implies that it may be necessary to express compounds with multiple inhibitory activities or a combination of different types of inhibitors.

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Received in November, 2007. Accepted for publication in June, 2008.