




# Biochemical Properties and Catalytic Specificity of a Novel Neutral Serine Peptidase Secreted by Fungus *Pyrenochaetopsis* sp.

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Received: 22 May 2018 / Accepted: 24 August 2018 /

Published online: 4 September 2018

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## Abstract

The fungal genus *Pyrenochaetopsis* has received particular attention because of its different lifestyles, such as numerous plant pathogenic, saprophytic, and endophytic species. Its ability to infect plant cells relies heavily upon secreted peptidases. Here, we investigated the biochemical properties and catalytic specificity of a new serine peptidase secreted by the filamentous fungus *Pyrenochaetopsis* sp. We found that while this neutral serine peptidase displayed optimal activity at a pH of 7.0 and temperature of 45 °C, it tolerated a wide range of pH conditions and temperatures lower than 45 °C. Its peptidase activity was depressed by some metallic ions (such as aluminum, cobalt, and copper (II) chloride) and enhanced by others (such as sodium, lithium, magnesium, potassium, calcium, and manganese). Lastly, the enzyme showed the greatest specificity for non-polar amino acids, particularly leucine and isoleucine, and moderate specificity for basic and neutral polar amino acids. It displayed the least specificity for acidic residues.

**Keywords** Enzyme · Fungal protease · Proteolytic enzymes · Pathogen · *Pyrenochaetopsis*

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## Introduction

*Pyrenochaetopsis* sp. represent a fungal genus comprising species with different lifestyles, such as numerous plant pathogenic, saprophytic, and endophytic species [1]. It is well-known that the secretion of proteolytic enzymes is an important strategy for exploring the nutritional resources found in the growth environment of the microorganism. In addition, these enzymes play an important role in the definition of the microbial lifestyle, being essential in decomposing biomass (saprophytic) and for the establishment in host organisms (pathogenic) [2]. In cases of pathogenicity, a comprehensive characterization of the catalytic function, biochemical properties, and substrate specificities of these peptidases is required to develop effective strategies for controlling infections [3].

Among these fungal proteolytic enzymes, serine peptidases (E.C. 3.4.21) are the most abundant group of endopeptidases. They hydrolyze peptide bonds under a wide range of pH conditions and they have broad substrate specificity. This group of peptidases is characterized by a dependence on serine residues, and they typically act upon a catalytic triad consisting of His, Ser, and Asp [4, 5].

*Pyrenochaetopsis* is a still poorly known fungal genus with few references to enzymes secreted by these fungi. By means of this, it is possible to understand the scientific contribution of this work, since the study of proteolytic enzymes is a scientific novelty to be described from this fungal genus. To our knowledge, this is the first characterization of the biochemical and catalytic properties of a serine peptidase produced by a representative of the genus *Pyrenochaetopsis*.

Studying the specificity of these peptidases improves our understanding of the molecular basis of the substrate preference of the peptidases [5, 6]. This research provides important mechanistic information about the degradation of a large variety of proteins, and might help identify strategies for controlling pathogenic agents that threaten crops and animals worldwide.

In this study, we purified a novel serine peptidase from the filamentous fungus *Pyrenochaetopsis* sp., and characterized its biochemical properties and substrate specificities.

## Materials and Methods

### Microorganisms, Identification, and Maintenance Media

The filamentous fungus *Pyrenochaetopsis* sp. was isolated from soil samples. Genomic DNA was extracted, quantified, and sequenced using primer pairs for the internal transcribed spacer (ITS).

The fungal strain was maintained on potato dextrose agar slants for 168 h at 30 °C to allow for complete growth. After that, the fungus was subjected to submerged bioprocess to produce peptidase.

### Submerged Bioprocess (Smb)

Erlenmeyer flasks (250 mL) were filled with 50 mL of fermentation media (0.7%  $\text{KH}_2\text{PO}_4$ , 0.2%  $\text{K}_2\text{HPO}_4$ , 0.01%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.05% citrate $\cdot 2\text{H}_2\text{O}$ , 0.1% yeast extract, 0.01%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , pH 6.0) [7, 8], and supplemented with 2% (w/v) crushed feathers. The

fermentation media was autoclaved for 15 min at 121 °C, and inoculated using 1 mL of a mycelial suspension (water) in standardized fungal growth media for 168 h at 30 °C.

Based on preliminary experiments (data not shown), we found that the highest peptidase production occurred with 120 h of cultivation at 30 °C and 120 rpm. The cultures were therefore treated according to this protocol. The media was subsequently removed, filtered with Whatman paper No. 1, and centrifuged at 8000 ×g for 20 min at 4 °C. The supernatant was then used for chromatographic study.

## Total Protein Quantification

Protein quantification was performed according to the Bradford [9] method using bovine serum albumin as a standard.

## Proteolytic Activity and Concentration of Crude Enzymatic Extract

Protease activity was measured using a caseinolytic assay, and we performed the enzymatic reaction according to Silva et al. [10]. Briefly, we added 1 mL of 1% (w/v) casein (Sigma) dissolved in 0.05 M NaH<sub>2</sub>PO<sub>4</sub> (monobasic) (pH 6.5) to 0.1 mL of the enzyme solution for 120 min at 45 °C. The reaction was stopped with the addition of 0.6 mL of 10% trichloroacetic acid, and the samples were centrifuged at 10,000 ×g for 15 min at 25 °C. Proteolysis quantification was performed by determining the absorbance of the supernatant at 280 nm using a spectrophotometer. The proteolytic activity unit (U/mL) was defined as the amount of enzyme required to liberate 1 μM tyrosine in 1 min under the assay conditions [8, 10–13].

Using a similar methodology as Silva et al. [10], the crude enzyme extract from the SmB was concentrated and partially purified using a hollow fiber membrane (10 kDa) coupled to a flex stand system (GE Healthcare) at 4 °C.

## Chromatography

Purification was performed using size-exclusion chromatography, followed by ion-exchange chromatography. Briefly, a Sephadex G-50 resin column (100 cm × 4 cm) was equilibrated with 1.5 column volumes of 0.05 M acetate buffer supplemented with 50 mM NaCl (pH 5.0). After this equilibration, the concentrated crude protein extract from the SmB was applied to the column. A flow rate of 0.6 mL/min was established, and fraction volumes of 5 mL were collected and evaluated by the caseinolytic assay. Protein concentrations were determined using a spectrophotometer (280 nm absorbance), and then subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [14]. Afterwards, fractions with similar electrophoretic profiles were pooled and dialyzed in 0.05 M acetate buffer (pH 4.5).

We then subjected the samples to cation-exchange chromatography using a Source 15S resin column (10 cm × 1 cm) coupled to an Äkta purifier (GE Healthcare). The resin column was first equilibrated with 10 column volumes of buffer A (0.05 M acetate buffer, pH 4.5) and a flow rate of 1 mL/min was maintained using buffer A and buffer B (0.05 M acetate buffer, 0.2 M NaCl). Each fraction volume was 2.5 mL.

After chromatography, the collected fractions were analyzed for total protein content (280 nm absorbance). The caseinolytic assay was performed and electrophoretic

profiles were determined using 12% SDS-PAGE [14]. Silver nitrate gel staining was performed according to the methods of See and Jackowski [15]. Molecular weight of the proteins was estimated from the gel bands using Image Lab 3.0 software (Bio-Rad).

## Mass Spectrometry

Peptidase identification was achieved by sequencing the peptide fragments using mass spectrometry. Briefly, we excised the protein bands of interest from the 12% SDS-PAGE gels stained with Coomassie blue G-250. The bands were treated with a solution of 50% (v/v) acetonitrile in 0.1 M  $\text{NH}_4\text{HCO}_3$ , pH 7.8. Samples were then dehydrated with 100% acetonitrile for 10 min, followed by an additional drying step in a speed vacuum concentrator.

Trypsin digestion was performed using 10  $\mu\text{L}$  of a 20 ng/ $\mu\text{L}$  stock of modified trypsin (T6567—Sigma Aldrich) in 0.1 M  $\text{NH}_4\text{HCO}_3$  (pH 7.8) for 18 h at 37 °C. The reaction was stopped by the addition of 5  $\mu\text{L}$  of formic acid. Elution and desalination of the trypsinized peptides were achieved using ZipTip pipette tips filled with reverse-phase resin (POROS R2; Perceptive Biosystems, Foster City, CA, USA) [10–12]. First, 10  $\mu\text{L}$  of resin was equilibrated using 300  $\mu\text{L}$  of 0.2% formic acid. The peptides were then eluted using 50  $\mu\text{L}$  of a 60% methanol/5% formic acid solution.

After elution, samples were concentrated using speed vacuum, resuspended in  $\alpha$ -cyano-4-hydroxycinnamic acid matrix solution (5 mg/mL) and loaded into a matrix-assisted laser desorption ionization (MALDI) target. The analysis was performed with a MALDI time-of-flight (TOF) mass spectrometer (Axima Performance; Kratos-Shimadzu, Manchester, UK). Using the MASCOT search engine (version 2.2.04), the database search parameters specified were trypsin hydrolysis, maximal number of one missed cleavage, and methionine oxidation as variable modification.

## Functional Biochemical Characterization

The catalytic properties of the enzyme were investigated using hydrolysis of the fluorescence resonance energy transfer (FRET) substrate, Abz-KLRSSKQ-EDDnp. The reaction was carried out in 0.1 M HEPES buffer (pH 7.0) at 45 °C and quantified using a Lumina fluorescence spectrometer (Thermo Scientific) at  $\lambda_{\text{ex}}=320$  nm and  $\lambda_{\text{em}}=420$  nm in a Peltier system 4-Position Cell Holder Fluorescence with quartz cuvettes (10 mm optical path length).

## Effect of pH and Temperature on the Activity and Stability of the Peptidase

The optimal pH for robust peptidase activity was determined using the following buffers at a concentration of 0.1 M each: acetate (pH 4.5 and 5.0), MES (pH 5.5, 6.0, and 6.5), HEPES (pH 7.0, 7.5, and 8.0), BICINE (pH 8.5 and 9.0), and CAPS (pH 9.5, 10.0, and 10.5). The stability of the peptidase in different pH conditions was determined by incubating the enzyme for 1 h at 25 °C in solutions ranging from pH 4.5 to 10.5. Finally, the optimal temperatures for peptidase activity and stability were assessed. The optimal activity was tested at temperatures of 35 to 70 °C and the thermal stability was tested at 40 to 60 °C. Each incubation was performed for 5, 30, or 60 min.

## Effect of Inhibitors and Metallic Ions on Peptidase Activity

The effect of different ions on peptidase activity was investigated using the following chloride salts: NaCl, LiCl, MgCl<sub>2</sub>, KCl, CaCl<sub>2</sub>, MnCl<sub>2</sub>, BaCl<sub>2</sub>, AlCl<sub>3</sub>, CoCl<sub>2</sub>, and CuCl<sub>2</sub>. The following peptidase inhibitors were examined as well: iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF), and ethylenediaminetetraacetic acid (EDTA). All the metallic ions and inhibitors were used at a final concentration of 5 mM.

## Denaturing Effect of Surfactants, Urea, DTT, and Guanidine on Peptidase Activity

To assess the effects of denaturing agents on peptidase activity and to determine the importance of intermolecular forces in enzyme folding, we incubated the peptidase with ionic surfactants (cetyltrimethylammonium bromide [CTAB] and sodium dodecyl sulfate [SDS]) and nonionic surfactants (Tween 20 and Triton X-100) at concentrations ranging from 0.02–1%. Additionally, we investigated the effect of urea, dithiothreitol (DTT), and guanidine at various concentrations (10, 25, 50, 100, and 150 mM).

## Determination of the Concentration and Specificity of the Peptidase

Active peptidase at a concentration of 2.35 mM was quantified by active-site titration [10–12, 16] using PMSF as a serine peptidase inhibitor. To determine the catalytic specificity for the S<sub>3</sub>, S<sub>2</sub>, S<sub>1</sub>, S'<sub>1</sub>, S'<sub>2</sub>, and S'<sub>3</sub> subsites, we assessed enzymatic kinetics at pH 7.0 in 0.1 M HEPES buffer at 45 °C. The following FRET substrates with residue replacements indicated by “X” were used: Abz-XLRSSKQ-EDDnp (P<sub>3</sub>), Abz-KXRSSKQ-EDDnp (P<sub>2</sub>), Abz-KLXSSKQ-EDDnp (P<sub>1</sub>), Abz-KLRXSKQ-EDDnp (P'<sub>1</sub>), Abz-KLRSXKQ-EDDnp (P'<sub>2</sub>), and Abz-KLRSXQ-EDDnp (P'<sub>3</sub>). Plotting was performed using the Michaelis–Menten equation, and the kinetic parameters  $K_M$  and  $V_{max}$  were obtained using nonlinear regression hydrolysis data in GraphPad Prism software (version 5.0).

To examine the scissile bond of the hydrolyzed peptides and infer catalytic specificity, we isolated peptide fragments using analytical HPLC and determined their molecular masses using an LCMS-2020 equipped with an ESI-probe (Shimadzu, Tokyo, Japan).

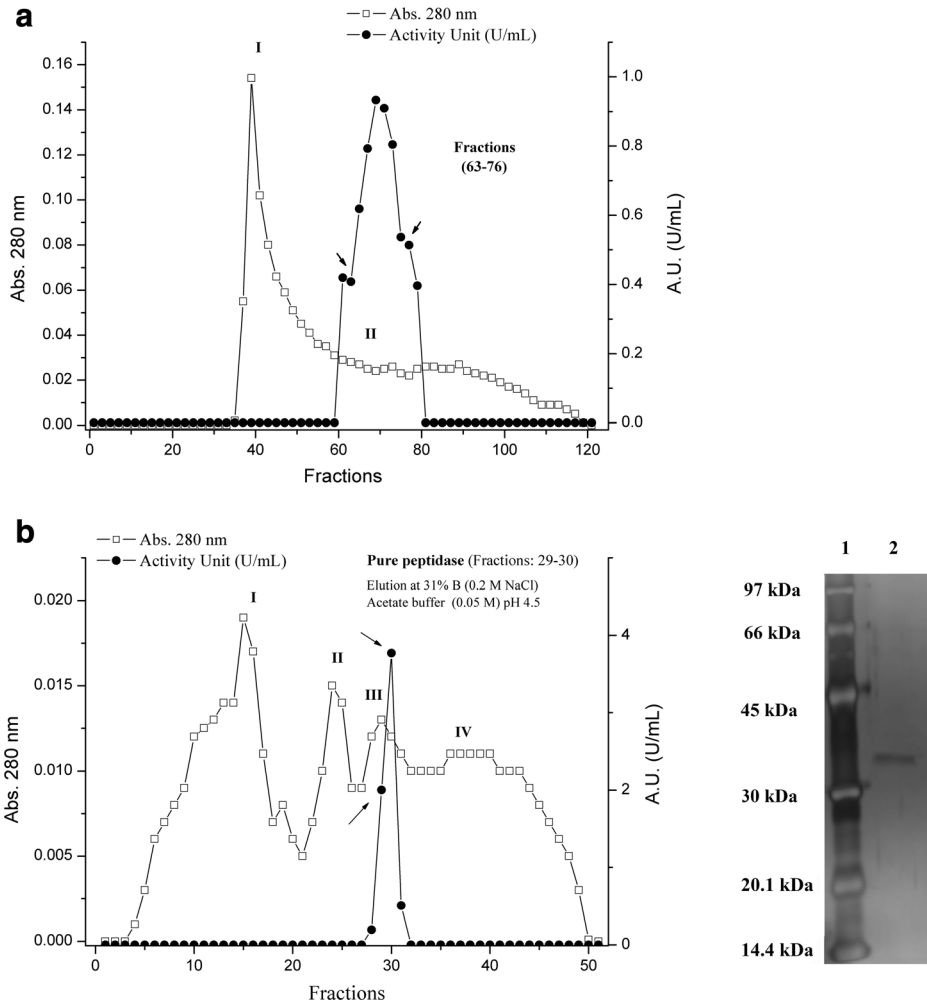
## Statistical Analyses

GraphPad Prism software (version 5.0) was used to calculate statistical variance (ANOVA). Differences between samples were considered statistically significant if  $p < 0.05$ .

## Results

### Peptidase Purification

Chromatographic assays were performed using size-exclusion chromatography and ion-exchange chromatography. Crude enzymatic extracts were applied to Sephadex G-50 columns. The elution profile exhibited one protein peak (peak I) and subsequent range fractions (II). The fractions were tested for proteolytic activity, and fractions 63–76 were selected for further experiments (Fig. 1A).



**Fig. 1** Chromatographic profiles of the peptidase after gel filtration on **A** Sephadex G-50 resin and **B** ion-exchange Source 15S resin and SDS-PAGE (12%) of the pure peptidase. Lane 1: molecular weight marker; lane 2: pure peptidase

For the second purification step, we used Source 15S. The chromatographic profile showed four protein peaks (absorbance at 280 nm), and we obtained pure peptidase from peak III, fractions 29–30, which we eluted at 31% in buffer B (Fig. 1B). According to the purification summary and analysis by SDS-PAGE, we obtained a yield of 3.5%, purification fold of seven times (Table 1). The pure enzyme had a molecular mass of approximately 35 kDa (Fig. 1B).

### Mass Spectrometry

Identification of the peptidase secreted by *Pyrenochaetopsis* sp. was performed by tryptic digestion and mass spectrometry analysis. The amino acid sequence obtained from analysis of this peptidase was KCVYWGGPVTVSNTKNYGRQ. We found that this peptidase did not display homology with other known peptidases (score < 60).

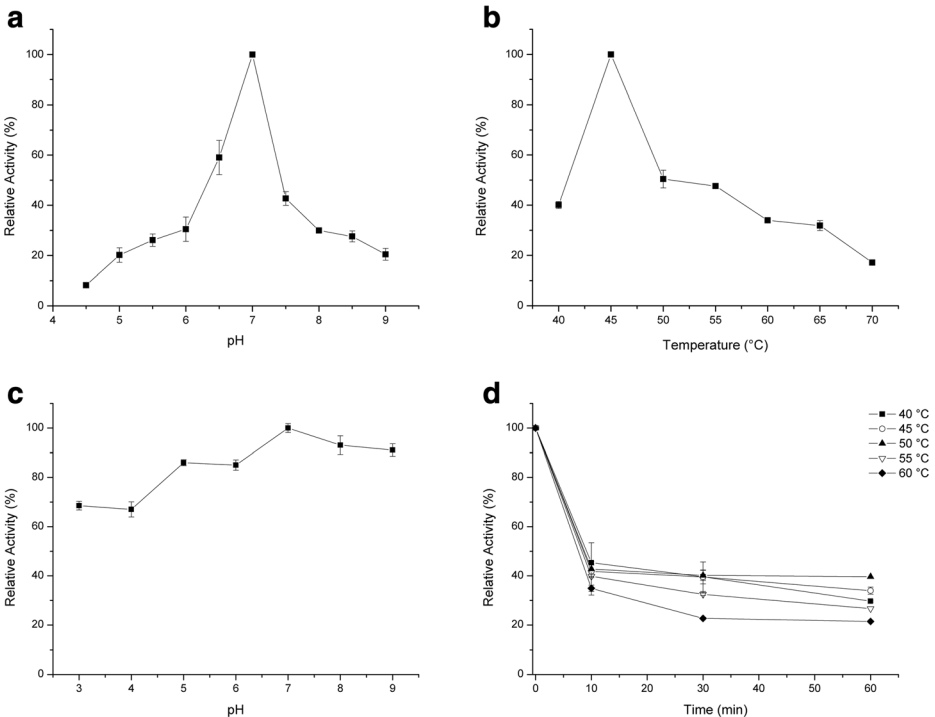
**Table 1** Summary of the purification of serine peptidase secreted by *Pyrenochaetopsis* sp.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract (SmB)	3912.0	41.0	95.5	1.0	100.0
Concentrated extract	3687.0	37.0	100.0	1.0	94.0
Sephadex G-50	2410.0	6.8	354.5	3.7	61.5
Source 15S	136.0	0.2	680.0	7.0	3.5

### Effect of pH and Temperature on the Activity and Stability of the Peptidase

The highest activity for this peptidase was observed at pH 7.0, and this activity level is reduced to 60 and 40% at pH 6.5 and 7.5, respectively (Fig. 2A). With regard to the effect of temperature, we detected maximal enzymatic hydrolysis at 45 °C. A progressive loss of proteolytic performance occurred at higher temperatures. For example, enzyme activity decreased by 50% at 50 °C (Fig. 2B).

Incubating the peptidase for 1 h at 25 °C in buffers of varying pH also influenced enzymatic stability. We observed that the enzyme could tolerate buffers of pH 3 and pH 4, which reduced its activity to 70% of its maximal activity. The highest stability was observed between pH 7 and 9 (Fig. 2C). Consistent with these results, in the thermal stability analysis, we found that

**Fig. 2** Effects of **A** pH and **B** temperature on the activity, **C** pH stability, and **D** thermal stability of the peptidase

the enzyme activity was reduced to 40% by 60-min incubation at temperatures between 40 °C and 50 °C. Temperatures higher than this severely reduced catalytic performance (Fig. 2D).

### Modulator Effect of Inhibitors and Metallic Ions on Peptidase Activity

Incubating the peptidase in the presence of metallic ions either increased or decreased catalytic activity, depending on the ions evaluated (Table 2). Some ions improved proteolytic activity by approximately 10–20%, such as sodium (21%), lithium (18%), magnesium (15%), potassium and calcium (12%), and manganese (10%). Compared to its effect on enzyme activity in the control, barium did not display a statistically significant effect on the catalytic activity of the peptidase ( $p = 0.11$ ). In contrast, other metallic ions inhibited peptidase activity, such as aluminum (14%), cobalt (45%), and copper (II). Copper (II) chloride, displayed particularly a negative modulation, resulting in a 96.5% reduction in proteolytic activity.

Additionally, using different inhibitors, the peptidase subclass was identified (Table 2). The 77% decrease in catalytic activity caused by PMSF, an inhibitor of serine peptidases, suggested that the purified enzyme was a serine peptidase. Additionally, the 40% reduction in peptidase activity in the presence of EDTA indicated the importance of metallic ions for the functioning of this enzyme.

### Effect of Urea, DTT, Guanidine, and Surfactants on Peptidase Activity

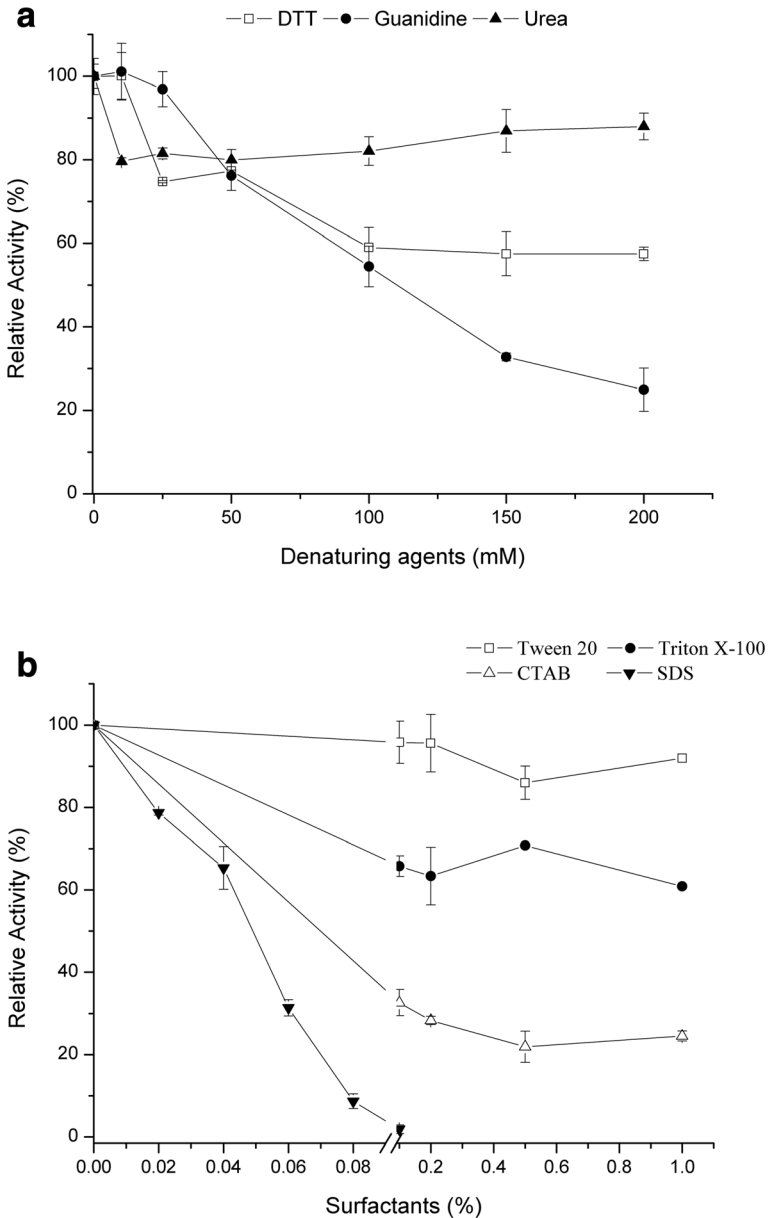
The effect of denaturing agents on peptidase activity provided important insights into the intermolecular forces required for proper folding of the enzyme. Specifically, we found that guanidine, DTT, and urea depressed relative activity of the peptidase (Fig. 3A). While all three compounds disrupted protein folding and depressed the enzyme, the negative effect exerted by urea was less than that exerted by guanidine and DTT. At a denaturant concentration of 100 mM, the peptidase exhibited 55% activity in the presence of DTT and guanidine, and 80% activity in the presence of urea. Furthermore, at a denaturant concentration of 200 mM, the overall peptidase activity was 57 and 25% in the presence of DTT and guanidine, respectively.

The protein denaturation caused by surfactants was more pronounced in the presence of ionic surfactants (CTAB and SDS) than in the presence of nonionic surfactants (Tween 20 and

**Table 2** Effect of metallic ions and inhibitors on peptidase activity

Metallic ions/inhibitors (5 mM)	Relative activity (%)
None	100.0 ± 0.2
Sodium chloride (NaCl)	121.0 ± 6.0
Lithium chloride (LiCl)	118.0 ± 8.0
Magnesium chloride (MgCl <sub>2</sub> )	115.0 ± 2.0
Potassium chloride (KCl)	112.0 ± 3.0
Calcium chloride (CaCl <sub>2</sub> )	112.0 ± 1.0
Manganese chloride (MnCl <sub>2</sub> )	110.0 ± 1.0
Barium chloride (BaCl <sub>2</sub> )	107.0 ± 3.5
Aluminum chloride (AlCl <sub>3</sub> )	86.0 ± 5.5
Cobalt chloride (CoCl <sub>2</sub> )	55.0 ± 1.0
Copper chloride (CuCl <sub>2</sub> )	3.5 ± 0.2
IAA	85.0 ± 2.0
EDTA	60.0 ± 8.0
PMSF	23.0 ± 0.2





**Fig. 3** Effects of **A** denaturing agents and **B** surfactants on the activity of the peptidase

Triton X-100). In particular, nonionic surfactants were responsible for only a minor reduction in protein folding, as indicated by approximately 90 and 60% catalytic activity exhibited in the presence of 1% Tween 20 and 1% Triton X-100, respectively (Fig. 3B).

In general, the denaturing effect of anionic (SDS) and cationic (CTAB) surfactants typically involves disruption of hydrophobic intramolecular interactions in the enzyme, consequently inhibiting proteolysis. As expected, we observed that the catalytic activity of the peptidase

reduced to 32% in the presence of 0.1% CTAB. Furthermore, its activity was lost entirely in the presence of 0.1% SDS.

### Characterizing the Specificity of the Serine Peptidase

The serine peptidase displayed a wide preference for nonpolar amino acids, in addition to a moderate acceptance for basic and neutral polar amino acids for most catalytic subsites examined. The peptidase also showed low catalysis for acidic amino acids (Asp and Glu) and nonpolar amino acids (Trp). In our experiments, we observed no hydrolysis or high  $K_M$  value, in addition to the low catalytic efficiency ( $k_{cat}/K_M$ ) for these amino acids.

For the  $S_1$  subsite, we determined the values for the following nonpolar amino acids: leucine,  $333.5 \pm 8.5 \text{ mM}^{-1} \text{ s}^{-1}$ ; isoleucine,  $200 \pm 3.5 \text{ mM}^{-1} \text{ s}^{-1}$ ; phenylalanine,  $154 \pm 3.2 \text{ mM}^{-1} \text{ s}^{-1}$ ; and methionine,  $132.5 \pm 7.5 \text{ mM}^{-1} \text{ s}^{-1}$ . For the basic amino acids, values were as follows: arginine,  $150 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$  and lysine,  $128.5 \pm 3.5 \text{ mM}^{-1} \text{ s}^{-1}$ . For the neutral polar amino acids, values were as follows: tyrosine,  $266.5 \pm 3.5 \text{ mM}^{-1} \text{ s}^{-1}$  and asparagine,  $200 \pm 3.5 \text{ mM}^{-1} \text{ s}^{-1}$ . For the  $S_2$  subsite, basic amino acid values were as follows: arginine,  $143 \pm 7 \text{ mM}^{-1} \text{ s}^{-1}$ ; histidine,  $180 \pm 5.7 \text{ mM}^{-1} \text{ s}^{-1}$ ; and lysine,  $133.5 \pm 17 \text{ mM}^{-1} \text{ s}^{-1}$ . The nonpolar amino acid value for leucine was  $150 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$ . For the  $S_3$  subsite, nonpolar amino acid values were as follows: leucine,  $1100 \pm 26 \text{ mM}^{-1} \text{ s}^{-1}$ ; valine,  $800 \pm 7.7 \text{ mM}^{-1} \text{ s}^{-1}$ ; and glutamine,  $266.5 \pm 12 \text{ mM}^{-1} \text{ s}^{-1}$ . Basic amino acid values were as follows: histidine,  $300 \pm 4.2 \text{ mM}^{-1} \text{ s}^{-1}$  and lysine,  $150 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$ . The neutral polar amino acid value for tyrosine was  $444.5 \pm 13 \text{ mM}^{-1} \text{ s}^{-1}$  (Table 3).

The  $S'$  subsites ( $S'_1$ ,  $S'_2$ , and  $S'_3$ ) displayed the highest preference for nonpolar amino acids, followed basic and neutral polar amino acids. For the  $S'_1$  subsite, we determined the values for the following amino acids: isoleucine,  $1250 \pm 17 \text{ mM}^{-1} \text{ s}^{-1}$ ; phenylalanine,  $384.5 \pm 3 \text{ mM}^{-1} \text{ s}^{-1}$ ; valine,  $250 \pm 13.5 \text{ mM}^{-1} \text{ s}^{-1}$ ; and arginine,  $233.5 \pm 16 \text{ mM}^{-1} \text{ s}^{-1}$ . For the  $S'_2$  subsite, we determined the values for the following amino acids: glutamine,  $625 \pm 19 \text{ mM}^{-1} \text{ s}^{-1}$ ; glycine,  $440 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$ ; phenylalanine,  $250 \pm 15 \text{ mM}^{-1} \text{ s}^{-1}$ ; methionine,  $186 \pm 3.7 \text{ mM}^{-1} \text{ s}^{-1}$ ; asparagine,  $416 \pm 2.8 \text{ mM}^{-1} \text{ s}^{-1}$ ; and arginine,  $171 \pm 29 \text{ mM}^{-1} \text{ s}^{-1}$ . For the  $S'_3$  subsite, we determined the values for the following amino acids: isoleucine,  $227 \pm 8 \text{ mM}^{-1} \text{ s}^{-1}$ ; methionine,  $180 \pm 3 \text{ mM}^{-1} \text{ s}^{-1}$ ; lysine,  $150 \pm 9 \text{ mM}^{-1} \text{ s}^{-1}$ ; and threonine,  $138 \pm 4.5 \text{ mM}^{-1} \text{ s}^{-1}$  (Table 4).

### Discussion

In this study, we describe the purification, identification, and biochemical characterization of a peptidase produced by *Pyrenochaetopsis* sp. We performed two rounds of chromatography and concentration protocols to obtain high yield and purity of this enzyme. In particular, the use of size-exclusion chromatography followed by ion-exchange chromatography proved a successful method for extracting this enzyme with extremely high purity and a final yield greater than that described in other studies of fungal peptidases [4, 17]. The molecular mass of the peptidase (estimated to be 35 kDa) was similar to other serine peptidases from different fungal species such as *Aspergillus fumigatus* [4], *Penicillium waksmanii* [18], and *Aspergillus clavatus* [19], which range from 32 to 35 kDa. Interestingly, the peptidase described in our study did not demonstrate high homology to other known proteolytic enzymes. It should be noted that there are not many reports of peptidases isolated from *Pyrenochaetopsis* sp. This

**Table 3** Kinetic parameters for the hydrolysis of the series of FRET peptides (S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> subsites)

X	Abz-KLXSSKQ-EDDnp (P <sub>1</sub> )			Abz-KXRSSKQ-EDDnp (P <sub>2</sub> )			Abz-XLRSSKQ-EDDnp (P <sub>3</sub> )		
	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>K</i> <sub>M</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>M</sub> (mM <sup>-1</sup> s <sup>-1</sup> )	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>K</i> <sub>M</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>M</sub> (mM <sup>-1</sup> s <sup>-1</sup> )	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>K</i> <sub>M</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>M</sub> (mM <sup>-1</sup> s <sup>-1</sup> )
A	Not determined			0.3 ± 0.05	4.7 ± 0.6	64 ± 1	0.2 ± 0.025	7.5 ± 0.2	26.5 ± 0.8
D	No hydrolysis			No hydrolysis			0.08 ± 0.003	6.5 ± 1.5	12.5 ± 0.5
E	No hydrolysis			No hydrolysis			0.15 ± 0.01	5.5 ± 0.4	27 ± 0.7
F	0.12 ± 0.02	0.78 ± 0.1	154 ± 3.2	0.12 ± 0.008	3.1 ± 0.25	39 ± 0.5	0.2 ± 0.01	7.0 ± 0.1	28.5 ± 1
H	0.20 ± 0.007	6.5 ± 0.3	31 ± 1.5	0.09 ± 0.006	0.5 ± 0.02	180 ± 5.7	0.36 ± 0.015	1.2 ± 0.2	300 ± 4.2
I	0.08 ± 0.012	0.4 ± 0.07	200 ± 3.5	0.10 ± 0.008	3.4 ± 0.04	29.5 ± 0.4	0.09 ± 0.002	0.65 ± 0.04	138.5 ± 9
K	0.18 ± 0.003	1.4 ± 0.3	128.5 ± 3.5	0.04 ± 0.06	0.3 ± 0.03	133.5 ± 17	0.06 ± 0.002	0.4 ± 0.02	150 ± 9
L	0.5 ± 0.05	1.5 ± 0.03	333.5 ± 8.5	0.06 ± 0.002	0.4 ± 0.02	150 ± 9	0.11 ± 0.0	0.1 ± 0.005	1100 ± 26
M	0.86 ± 0.03	6.5 ± 0.4	132.5 ± 7.5	0.18 ± 0.025	5.5 ± 0.07	33 ± 0.4	0.08 ± 0.008	1.0 ± 0.05	80 ± 4.8
N	0.10 ± 0.004	0.5 ± 0.01	200 ± 3.5	0.07 ± 0.003	1.5 ± 0.1	46.5 ± 3	0.21 ± 0.01	7.5 ± 0.1	28 ± 3
Q	0.20 ± 0.003	2.3 ± 0.3	87 ± 1.4	0.06 ± 0.001	0.9 ± 0.006	66.5 ± 0.4	0.04 ± 0.003	0.15 ± 0.001	266.5 ± 12
R	0.06 ± 0.002	0.4 ± 0.02	150 ± 9	0.05 ± 0.001	0.35 ± 0.01	143 ± 7	Not determined		
S	0.27 ± 0.06	18 ± 2	15 ± 1	0.12 ± 0.003	3.5 ± 0.15	34 ± 1.5	0.31 ± 0.005	2.0 ± 0.2	155 ± 11
T	0.09 ± 0.01	1.25 ± 0.07	72 ± 4	Not determined			0.36 ± 0.001	6.0 ± 0.01	60 ± 3
V	No hydrolysis			0.06 ± 0.003	1.2 ± 0.15	50 ± 6	0.12 ± 0.0	0.15 ± 0.0	800 ± 7.7
W	No hydrolysis			No hydrolysis			0.13 ± 0.01	8.8 ± 0.03	15 ± 0.6
Y	0.4 ± 0.003	1.5 ± 0.2	266.5 ± 3.5	0.07 ± 0.001	1.8 ± 0.05	39 ± 3.5	0.2 ± 0.02	0.45 ± 0.06	444.5 ± 13

Hydrolysis conditions: 100 mM HEPES buffer pH 7.0 at 45 °C. Values are the average of three independent experiments ± standard deviations. The peptidase showed, in general, two hydrolysis points at X<sub>1</sub>S/R<sub>1</sub>S and S<sub>1</sub>K. However, all the substrates presented a notorious predominance of cleavage at X<sub>1</sub>S/R<sub>1</sub>S

**Table 4** Kinetic parameters for the hydrolysis of the series of FRET peptides (S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> subsites)

X	Abz-KLRXSKQ-EDDnp (P <sub>1</sub> )			Abz-KLRSSXQ-EDDnp (P <sub>3</sub> )		
	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>K</i> <sub>M</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>M</sub> (mM <sup>-1</sup> s <sup>-1</sup> )	<i>k</i> <sub>cat</sub> (s <sup>-1</sup> )	<i>K</i> <sub>M</sub> (μM)	<i>k</i> <sub>cat</sub> / <i>K</i> <sub>M</sub> (mM <sup>-1</sup> s <sup>-1</sup> )
A	0.15 ± 0.01	1.15 ± 0.07	130.5 ± 8	0.21 ± 0.01	6.3 ± 0.5	33.5 ± 2.6
D	No hydrolysis			0.1 ± 0.006	1.15 ± 0.04	87 ± 3
E	No hydrolysis			0.07 ± 0.005	3 ± 0.01	23.5 ± 1
F	0.05 ± 0.001	0.13 ± 0.01	384.5 ± 3	0.08 ± 0.01	1.6 ± 0.001	50 ± 3
G	No hydrolysis			0.11 ± 0.001	440 ± 9	17 ± 0.5
H	No hydrolysis			0.47 ± 0.02	2.9 ± 0.07	48 ± 0.7
I	0.05 ± 0.003	0.04 ± 0.001	1250 ± 17	0.14 ± 0.02	0.22 ± 0.07	227 ± 8
K	0.22 ± 0.003	3.2 ± 0.05	69 ± 2	0.05 ± 0.0	0.4 ± 0.02	150 ± 9
L	No hydrolysis			0.06 ± 0.0	1.0 ± 0.001	60 ± 0.7
M	0.08 ± 0.009	0.5 ± 0.01	160 ± 7	0.09 ± 0.01	0.5 ± 0.003	180 ± 3
N	No hydrolysis			Not determined		
P	No determined			0.09 ± 0.02	1.8 ± 0.1	50 ± 3
Q	0.1 ± 0.003	1.5 ± 0.0015	66.5 ± 6	0.07 ± 0.0	0.8 ± 0.007	87.5 ± 0.5
R	0.07 ± 0.008	0.3 ± 0.009	233.5 ± 16	0.09 ± 0.01	5.2 ± 0.4	17.5 ± 1.5
S	0.06 ± 0.002	0.4 ± 0.02	150 ± 9	0.24 ± 0.04	5 ± 0.01	48 ± 1
T	0.08 ± 0.001	0.56 ± 0.015	143 ± 18	0.09 ± 0.01	0.65 ± 0.02	138.5 ± 4.5
V	0.03 ± 0.003	0.12 ± 0.01	250 ± 13.5	0.08 ± 0.01	5.7 ± 0.8	14 ± 0.3
W	Not determined			0.63 ± 0.04	26 ± 1	24 ± 0.5
Y	0.18 ± 0.03	4.0 ± 0.15	45 ± 1.2	0.20 ± 0.08	4.8 ± 0.15	41.5 ± 1

Hydrolysis conditions: 100 mM HEPES buffer pH 7.0 at 45 °C. Values are the average of three independent experiments ± standard deviations. The peptidase showed, in general, two hydrolysis points at R<sub>1</sub>X/R<sub>1</sub>S and S<sub>1</sub>K. However, all the substrates presented a notorious predominance of cleavage at R<sub>1</sub>X/R<sub>1</sub>S, except Abz-KLR<sub>1</sub>FS<sub>1</sub>JKQ-EDDnp (12% R<sub>1</sub>F and 88% S<sub>1</sub>K), Abz-KLR<sub>1</sub>MS<sub>1</sub>JKQ-EDDnp (35% R<sub>1</sub>M and 65% S<sub>1</sub>K), Abz-KLR<sub>1</sub>VS<sub>1</sub>JKQ-EDDnp (16% R<sub>1</sub>V and 84% S<sub>1</sub>K), and Abz-KLR<sub>1</sub>YS<sub>1</sub>JKQ-EDDnp (17% R<sub>1</sub>Y and 83% S<sub>1</sub>K)

reinforces the importance of our study, which examined the biochemical and catalytic properties of this enzyme. Our results provide important details about a novel proteolytic enzyme possibly required for the pathogenicity of *Pyrenochaetopsis* sp.

To study the activity and stability of this enzyme, we characterized its optimal activity requirements, including its pH tolerance and thermal stability range. The enzyme secreted by *Pyrenochaetopsis* sp. showed an optimal activity at a higher temperature than the peptidase secreted by *P. waksmanii* [18]. The peptidase showed sensitivity to PMSF, which acts as a serine peptidase inhibitor and blocks the action of several fungal neutral/alkaline peptidases [4, 5, 18]. In the presence of certain metallic ions, we observed that some ions were required for optimal catalytic activity of the serine peptidase. Furthermore, EDTA inhibited the activity of the peptidase, which indicated that ions are required for the enzyme's function.

In contrast, other metallic ions such as aluminum, cobalt, and especially, copper (II) dropped the activity of the peptidase. Ions can promote chemical interaction into a protein or the active site of an enzyme. This interaction can either agonize or antagonize catalytic activity. Our results suggest that the copper (II) ion, which completely inhibited the function of the enzyme, might bind to the active site of the serine peptidase and inhibit its ability to cleave its normal substrates.

Other studies of fungal serine peptidases reported that some metallic ions inhibited catalytic activity, while others enhanced catalytic activity. Silva et al. [4] noticed a slight increase in proteolysis in the presence of potassium ions and a decrease in proteolysis in the presence of aluminum ions. Hajji et al. [19] described an increase in catalytic activity in the presence of magnesium and calcium ions, and dropped its relative activity in the presence of zinc, copper (II), and cobalt ions. Calcium, barium, potassium, and magnesium ions also promoted the activity of serine peptidases in *P. waksmanii* [18].

Protein folding is dependent on intramolecular forces, such as hydrogen, hydrophobic, ionic, and disulfide bonds. Some chemical agents can weaken or break these molecular interactions, inhibiting enzymatic activity. We therefore investigated the effect of DTT, urea, and guanidine on the activity of the peptidase. DTT is capable of reducing disulfide bonds, while urea and guanidine interfere with hydrogen bonds. Additionally, we examined the effect of incubating the peptidase with surfactants, which disrupt hydrophobic intramolecular interactions.

Our results demonstrated the importance of chemical interactions on protein arrangement. Surfactants (especially SDS and CTAB), guanidine, and DTT likely denatured the enzyme. Additionally, we showed that compared with guanidine, urea had only a minor influence on proteolytic activity. This may be due to a difference of charge between urea (a neutral chaotropic agent) and guanidine (a cationic chaotropic agent). Under the same molar concentrations, the cationic denaturing agent was more effective at disrupting hydrogen bonds in the enzyme.

The sensitivity of peptidases to surfactants has been described, mainly with respect to ionic surfactants. These studies have demonstrated the requirement for a hydrophobic core in the folding of this protein. Similar results describing denaturation caused by surfactants and DTT have also been reported in other studies [10, 12, 20].

In comparison to other fungal peptidases, this enzyme was more tolerant to DTT than the enzyme secreted by *P. chrysosporium* [12] and more tolerant to CTAB than the enzyme secreted by *Aspergillus terreus* [21]. Under DTT effect, at 100 mM, the peptidase described in this work maintained approximately 55% of enzymatic activity, whereas the peptidase of *P. chrysosporium* [12] showed less than 10% of remaining activity. Under the effect CTAB at 0.2%, the peptidase

secreted by *Pyrenochaetopsis* sp. showed approximately 30% of residual activity, whereas the peptidase secreted by *A. terreus* [21] practically lost all proteolytic activity.

Regarding enzyme specificity, we observed that, in general, the peptidase showed preference to basic, neutral polar and, especially, nonpolar amino acids.

To determine which nonpolar amino acids the enzyme has the highest preference for, we replaced several amino acids in a peptide substrate. This allowed us to determine these residues' requirement for proteolysis by this serine peptidase. Our results showed that the hydrolysis of the peptide bond in a protein or a peptide fragment was easily achieved by anchoring the enzyme on a nonpolar peptide sequence substrate. Importantly, the enzyme displayed the best catalytic performance when leucine and valine were anchored on the S<sub>3</sub> subsite, isoleucine on S'<sub>1</sub> subsite, and glutamine and glycine on the S'<sub>2</sub> subsite. This demonstrated the requirement for nonpolar amino acids at these subsites.

In other studies of fungal serine peptidases, similar results were obtained that indicated a substrate preference for nonpolar amino acids. In these cases, catalytic performance was higher than that observed with neutral polar or cationic/anionic residues as substrates. In particular, fungal serine peptidases have been reported to display the greatest affinity for nonpolar residues such as leucine and isoleucine [4, 22].

In general, this study described the purification of a neutral serine peptidase. We characterized the peptidase's biochemical properties. The enzyme was sensitive to guanidine, surfactants, and DTT. Its catalytic activity was inhibited by some metallic ions and enhanced by others. With regard to its specificity, the enzyme displayed a preference for nonpolar amino acids and a low affinity for acidic amino acids anchoring their subsites. Based on our biochemical analysis and the enzyme's broad ability to hydrolyze proteins, this peptidase is likely important for hydrolyzing proteins that provide nutrients to the fungus.

According to these biochemical characteristics, this enzyme can be an important tool to hydrolyze tissue proteins in animals and plants, or degradation of organic matter in saprophytic lifestyle [2]. Some fungi of this genus have been distinguished by infecting humans (*P. botulispورا*, *P. confluens*, *P. globosa*, and *P. microspora*) or by infecting plants (*P. setosissima* and *P. poae*) [23]. Future studies should evaluate the involvement of this enzyme in pathologies and the host-pathogen interaction.

**Funding Information** The authors would like to acknowledge the financial support provided by Fundação de Amparo à Pesquisa do Estado de São Paulo-FAPESP (process 2011/06986-0 and 2012/24703-8), Conselho Nacional de Desenvolvimento Científico e Tecnológico, and Instituto Nacional de Ciência e Tecnologia-Rede de Biotecnologia Farmacêutica.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare no financial or commercial conflict of interest.

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