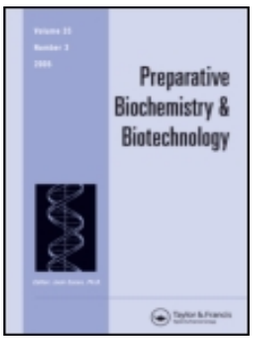

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Preparative Biochemistry and Biotechnology

ISSN: 1082-6068 (Print) 1532-2297 (Online) Journal homepage: https://www.tandfonline.com/loi/lpbb20

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To cite this article: Rafael Tage Biaggio, Ronivaldo Rodrigues da Silva, Nathalia Gonsales da Rosa, Rodrigo Simões Ribeiro Leite, Eliane Candiani Arantes, Tatiana Pereira de Freitas Cabral, Maria A. Juliano, Luiz Juliano & Hamilton Cabral (2016) Purification and biochemical characterization of an extracellular serine peptidase from *Aspergillus terreus*, Preparative Biochemistry and Biotechnology, 46:3, 298-304, DOI: 10.1080/10826068.2015.1031387

To link to this article: https://doi.org/10.1080/10826068.2015.1031387

Accepted author version posted online: 01 Apr 2015.
Published online: 14 Apr 2016.

PREPARATIVE BIOCHEMISTRY AND BIOTECHNOLOGY
2016, VOL. 46, NO. 3, 298–304
http://dx.doi.org/10.1080/10826068.2015.1031387

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Purification and biochemical characterization of an extracellular serine peptidase from *Aspergillus terreus*

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ABSTRACT

Peptidases are important because they play a central role in pharmaceutical, food, environmental, and other industrial processes. A serine peptidase from *Aspergillus terreus* was isolated after two chromatography steps that showed a yield of 15.5%. Its molecular mass was determined to be 43 kD, by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This peptidase was active between pH 5.0 to 8.0 and had maximum activity at pH 7.0, at 45°C. When exposited with 1 *M* of urea, the enzyme maintained 100% activity and used azocasein as substrate. The N-terminal (first 15 residues) showed 33% identity with the serine peptidase of *Aspergillus clavatus* ES1. The kinetics assays showed that subsite S_2 did not bind polar basic amino acids (His and Arg) nonpolar acidic amino acids (Asp and Glu). The subsite S_1 showed higher catalytic efficiency than the S_2 and S_3 subsites.

KEYWORDS

Aspergillus; enzyme kinetics; filamentous fungi; N-terminal sequence; protease; protein

Introduction

Peptidases belong to the class of hydrolases that catalyze the peptide bonds of proteins and peptides, and they play an important nutritional and regulatory role in different organisms. Extracellular peptidases act mainly in protein and polypeptide degradation, for production of energy and biosynthesis precursor by fungi.[1]

Peptidases are enzymes that occupy a central position due to their applications in both the physiological and commercial fields.[2] They comprise an enzyme group that represents a quarter of the worldwide sale of enzymes. These enzymes are applied in detergents, meat tenderizers, leather treatment, the dairy industry, waste treatment, and others.[3,4]

Hydrolyzed proteins are important products in the food and nutraceutical industries for two reasons. The first reason is that they promote improved nutrition, bioactivity, and functional properties of the food such as solubility, digestibility, sensory quality, and antioxidant capacity. The second reason is that the enzymatic hydrolysis has advantages over the chemical process, because the enzyme is more specific, more controllable, and it does not harm the environment.[5]

The genus *Aspergillus* is known for its ability to secrete high levels of enzyme to the environment, and several enzymes secreted by this genus have been widely used in the food and beverages industries for decades.[6]

Aspergillus terreus is known to produce extracellular enzymes with proteolytic activity. Previous findings indicate that metallopeptidases produced promote postsecretional proteolysis of high-molecular-mass cellulases into smaller enzymes of the cellulolytic complex.[7]

The aim of this work is to describe the biochemical characterization and kinetics parameters of a purified serine peptidase from *Aspergillus terreus*.

Experimental

Microorganism and maintenance

Aspergillus terreus was maintained on potato–dextrose agar slants at 30°C for 7 days. After this period, the slants were stored at 4°C. The spores were resuspended through addition of sterile water and quantified in a Neubauer chamber.

Submerged fermentation (SmF)

Peptidase production by submerged fermentation (SmF) was performed in Erlenmeyer flasks (250 mL) and consisted of 50 mL of liquid medium, containing 0.2% KH_2PO_4, 0.7% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract, 0.5% NaCl, and 0.5% casein, as reported in Tran and Nagano,[8] which used also the reagents citrate $\cdot 2H_2O$ 0.05%, gelatin 0.3% $CaCl_2 \cdot 2H_2O$, glucose 1%, and polypeptone 1%, at pH 7.4. The medium was adjusted to pH 6.0 and the flasks were autoclaved for 15 min at 121°C.

The SmF medium was inoculated with 5×10^6 spores/mL from *A. terreus* and incubated in a shaker at 30°C and 120 rpm, for 72 hr. The material was filtered with Whatman paper number 1 and centrifuged at 8000 × g for 20 min, at 4°C.

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Determination of proteolytic activity

Proteolytic activity was determined according to the protocol described by Sarath et al.,[9] with modifications (the original protocol used filtration in Whatman paper number 3).

The reaction mixture was composed of 1 mL of 1% (w/v) casein (Sigma) in HEPES buffer (50 m*M*, pH 7) and 0.2 mL of the enzyme solution. The reaction was carried out at 40°C and incubated for 60 min. The enzymatic reaction was stopped by the addition of 0.6 mL of 10% trichloroacetic acid (TCA). Reaction and blank tubes were centrifuged at 10,000 × g for 15 min, at 25°C. The absorbance of the supernatant for the reaction tubes was measured by spectrophotometer at 280 nm, against their respective blank tubes. Activity units (AU) were expressed as the amount of enzyme required to promote the liberation of 1 µmol tyrosine/min, under defined assay conditions.[9]

Biochemical studies were performed using 1% azocasein as a substrate, according to the protocol described by Ducros et al.[10] with modifications (that used substrate 0.3% and TCA 10% concentration). The reaction mixture consisted of 0.1 mL of purified enzyme, 0.1 mL of HEPES buffer (50 m*M*, pH 7), and 0.2 mL of 1% azocasein. The mixture was incubated at 40°C for 10 min, and the reaction was stopped by the addition of 0.8 mL 20% TCA. Reaction and blank tubes were centrifuged at 10,000 × g for 15 min at 25°C, and 0.8 mL of supernatant was harvested and transferred to a clean tube; then 0.9 mL of 1 *M* sodium hydroxide was added to the supernatant. The tubes were shaken and the absorbance was measured in a spectrophotometer at 440 nm and compared with the respective blank tubes. Activity units were defined as the amount of enzyme required to yield an increase of $0.001A_{440nm}$ under the conditions of the assay as described by Morita et al.[11] with modifications (Morita et al. used a wavelength of 340 nm).

Protein concentration was determined according to the Bradford method,[12] using bovine serum albumin as a standard.

Purification: Precipitation by ethanol, gel filtration, and ion exchange chromatography

The enzyme obtained by the fermentation process was precipitated and partially fractionated with ethanol 92.8°GL (about 70% v/v) at a ratio of 1:3 (enzymatic extract:ethanol). The material was maintained at −20°C overnight and then it was precipitated by centrifugation at 10,000 × g for 20 min, at 4°C. The precipitate was dissolved in sodium acetate buffer (50 m*M*, pH 5.5), named buffer A.

After being dissolved in buffer A, the precipitate was subjected to gel filtration in a Sephadex G-50 column (100 cm × 2.5 cm) previously equilibrated with 50 m*M* NaCl in buffer A, and the elution was performed with the same buffer. Fractions of 5 mL were collected at a flow rate of 0.5 mL/min. Protein content was measured at 280 nm and the proteolytic activities presented by the fractions were determined, as previously described. Fractions containing proteolytic activity were pooled and dialyzed in BICINE buffer (30 m*M*, pH 9), named buffer B.

The pooled dialyzed enzyme was subjected to ion-exchange chromatography on a Q-Sepharose column (15 cm × 1.5 cm) equilibrated with buffer B (30 m*M*, pH 9). Elution was performed with a gradient containing 0 to 500 m*M* of NaCl in buffer B. Fractions of 5 mL were collected at a flow rate of 0.5 mL/min. Protein content was measured at 280 nm and the proteolytic activity presented by the fractions was determined as previously described. Fractions containing proteolytic activity were pooled and dialyzed in ultrapure water at 4°C for 24 hr.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Certification of purity of the peptidase was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the protocol described by Laemmli.[13] The running gel was 12%, and the stacking gel, 4% polyacrylamide. Staining was performed according to Blum et al.[14]

Physical–chemical characteristics

Biochemical studies were determined using 1% azocasein as a substrate, according to the protocol described by Ducros et al.[10] with modifications.

Determination of pH and temperature effects

The effect of pH on the proteolytic activity of the purified peptidase was determined at different pH values, utilizing the following buffers: acetate (pH 4.5 and 5.0), MES (pH 5.5, 6 and 6.5), HEPES (pH 7, 7.5 and 8), BICINE (pH 8.5 and 9), and CAPS (pH 9.5, 10 and 10.5), all at final concentrations of 50 m*M* and using 1% azocasein as substrate at 40°C.

The influence of temperature on the activity of purified peptidase was investigated in the range of 25°C to 70°C, with 5°C increments. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7).

Determination of inhibitor effect

The mechanism of enzyme action was determined according to the protocol described by Dunn[15] with modifications (Dunn used the inhibitors E-64 for cysteine protease, pepstatin for aspartic protease, and 1,10-phenantroline for metalloprotease). The inhibitors iodoacetic acid (IAA), phenylmethylsulfonyl fluoride (PMSF), and ethylenediamine tetraacetic acid (EDTA) were used at a final concentration of 10 m*M*. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7) at 40°C.

Determination of surfactant effect

The effect of anionic sodium lauryl sulfate (SDS) and cationic cetyl trimethyl ammonium bromide (CTAB) surfactants on the proteolytic activity of the purified peptidase was investigated at percentages of 0.1, 0.25, 0.5, 0.75, and 1%. The peptidase was previously incubated with SDS and CTAB for 5 min, at 40°C. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7) at 40°C.

Determination of metal ion effect

The metal ion effect was determined at a final concentration of 5 m*M*. The peptidase was previously incubated with metal ions $CoCl_2$, KCl, LiCl, $AlCl_3$, $MgCl_2$, $BaCl_2$, $CaCl_2$, $MnCl_2$, $CuCl_2$, and NaCl for 5 min, at 40°C. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7) at 40°C.

Determination of reducing agent effect

The effect of the reducing agent dithiothreitol (DTT) on the proteolytic peptidase activity was investigated at the concentrations of 5, 10, 20, 40, 60, 80, and 100 m*M*. The peptidase was previously incubated with DTT for 5 min at 40°C. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7), at 40°C.

Determination of urea effect

The effect of urea on the proteolytic activity was investigated at concentrations of 50, 100, 250, 500, and 1000 m*M*. The peptidase was previously incubated with urea for 5 min, at 40°C. All experiments were performed using 1% azocasein as substrate in HEPES buffer (50 m*M*, pH 7), at 40°C.

Enzyme kinetics

The assays were performed in spectrofluorimeter using synthetic FRET peptides with mutations in amino acids residues in P_1, P_2, and P_3 positions of standard substrate Abz-KLRSSKQ-EDDnp. The substrate Abz-KLXSSKQ-EDDnp showed mutation in X (where X is D, E, F, H, I, M, R, S, T, and Y). The substrate in position P_2 (Abz-KXFSSKQ-EDDnp) showed mutation in X (where X is D, E, F, H, I, M, R, S, T, and Y). In P_3, the same substrate, Abz-XLRPSKQ-EDDnp, was also used, with mutations in X D, E, F, H, I, M, R, S, and T. The fluorescence was continuously measured and the excitation and emission wavelengths were 320 nm and 420 nm, respectively. The determination of kinetic parameters was performed under pseudo-first-order conduction ($[S] << k_M$) to hydrolysis of all substrates.[16] Data were plotted using the computation program GraphPad Prism, using the appropriate equation.

N-terminal amino acid sequence of the enzyme

The N-terminal sequence was determined using Protein Sequencer PPSQ-33A (Shimadzu Corporation, Kyoto, Japan). The PPSQ-33A system sequentially cleaves the N-terminal amino acids of proteins and peptides using Edman degradation. The PTH-amino acid obtained with Edman degradation (1967) is separated using high-performance liquid chromatography (HPLC) and is identified and quantified, compared to a previously quantified standard, with its retention times and ultraviolet (UV) absorption [17].

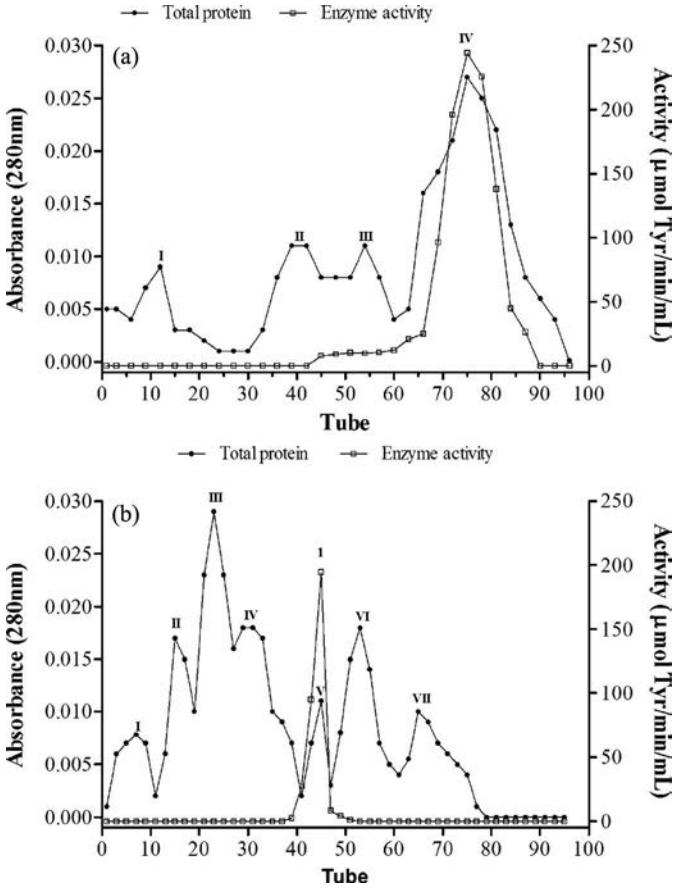

Figure 1. (a) Gel filtration G-50 Sephadex chromatography of the serine peptidase from *A. terreus*. Pooled fractions 35 to 72. The column was equilibrated and eluted with 50 m*M* sodium acetate buffer, pH 5.0, with 50 m*M* NaCl. (b) Elution profile ion exchange of the peptidase by *A. terreus* from the Q-Sepharose column. The enzyme was eluted with a linear gradient of NaCl (0 to 500 m*M*) in 50 m*M* bicine buffer, pH 8.0, at a flow rate of 1 mL/min (60 mL/hr).

Results and discussion

The purification process of the peptidase from *A. terreus* was performed in three steps and is presented in Table 1. In the first step, the peptidase was precipitated with ethanol and enzyme recovery was 90.4%, with high specific activity (2,735 U/mg of protein). In the second step, gel filtration in Sephadex G-50 was performed and the profile of elution using this resin showed four peaks of protein, and only peak IV showed proteolytic activity (Figure 1a).

We observed a reduction of the specific activity and recovery after gel filtration, when compared with the first step

Table 1. Purification summary of serine peptidase from *A. terreus* produced by submerged fermentation.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	151,200	140	1080	100	1
Precipitation	136,750	50	2735	90.4	2.5
Sephadex G-50	41,600	20	2080	27.5	1.9
Q-Sepharose	23,400	18	1300	15.5	1.2

(Table 1). This may be explained by the exposure of the peptidase to room temperature. Sampaio e Silva et al. also observed that the acid peptidase of *Aspergillus clavatus* lost activity during the purification process.[18]

The fraction containing pooled proteolytic activity was subjected to ion-exchange chromatography (Q-Sepharose). Seven peaks of protein were eluted and only peak V showed proteolytic activity (Figure 1b). The third step of the ion exchange was characterized by high specific activity of 1,300 U/mg of protein, and in this purification step, the peptidase was purified 1.2-fold with a recovery of 15.5% (Table 1).

The high specific activity observed in the third step of the ion-exchange chromatography may be explained because the chromatography process was in preserved peptidase at 4°C and also by the elimination of other proteins. Another possibility was the elimination of the compound that promotes peptidase inhibition.[19]

The peptidase showed only one band with approximate molecular mass of 43 kD in SDS-PAGE (Figure 2). In general, peptidases show microbial molecular mass of approximately 18 to 35 kD.[20]

The influence of pH on the activity of the peptidase showed that it is active between pH 4.5 and pH 10.5, but the maximum activity is at pH 7 (Figure 3a). Chakrabarti et al. observed that the peptidase of the fungus *A. terreus* IJIRA 6.2 showed wide activity (pH 4–12) with a maximum activity at pH 8.5.[21] Hussain et al. also reported that the serine peptidase of the fungus *A. terreus* showed optimum activity at pH 8.5, using casein as a substrate.[1] Another study demonstrated that a serine peptidase isolated from fungus *Hirsutella rhossiliensis* OWVT-1 showed optimum activity at pH 7.[22]

The effect of temperature on peptidase activity was measured between 25°C and 55°C, and showed maximum activity at 45°C (Figure 3b), and the peptidase was shown to be sensitive at increases in temperature above 45°C. The optimum temperature of the peptidase produced by fungus *H. rhossiliensis* OWVT-1 was determined to be at 40°C.[22] The serine peptidase of fungi *Aspergillus fumigatus* showed maximum activity at 37°C.[21] In another study, the optimum temperature of the fungi *A. terreus* isolated was 40°C.[1] Fungal peptidases show an optimum temperature range from 40°C to 60°C.[23]

Looking at the effect of inhibitors, PMSF reduced the peptidase activity by 99%, compared to the control (Table 2). Due to inhibition of peptidase by PMSF and the activity maintained in the presence of other inhibitors, the peptidase was classified as a serine peptidase. The increased activity of 15% in the presence of EDTA probably occurred due to adsorption of ions that can cause inhibition of peptidases. The other authors Stefanova et al. identified in *A. terreus* two different classes of peptidases: serine thiol dependent ones and metallopeptidases.[7]

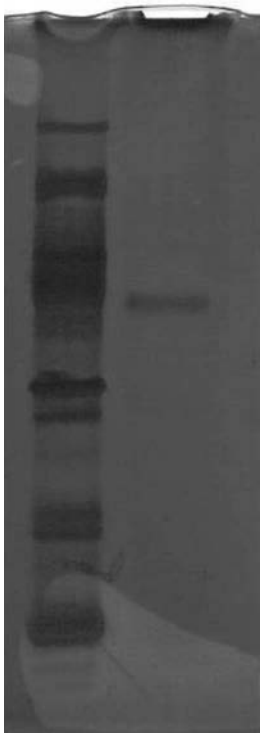

Figure 2. SDS-PAGE electrophoresis gel of a purified pool of ion-exchange chromatography from *A. terreus*. Lane 1: Standard molecular mass marker proteins (from the top): phosphorylase B (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonate dehydratase (30 kD), trypsin inhibitor (20 kD), and α-lactoalbumin (14.4 kD). Lane 2: purified peptidase. The gel was stained with silver nitrate and analyzed by software Image Lab version 3.0 (Bio-Rad).

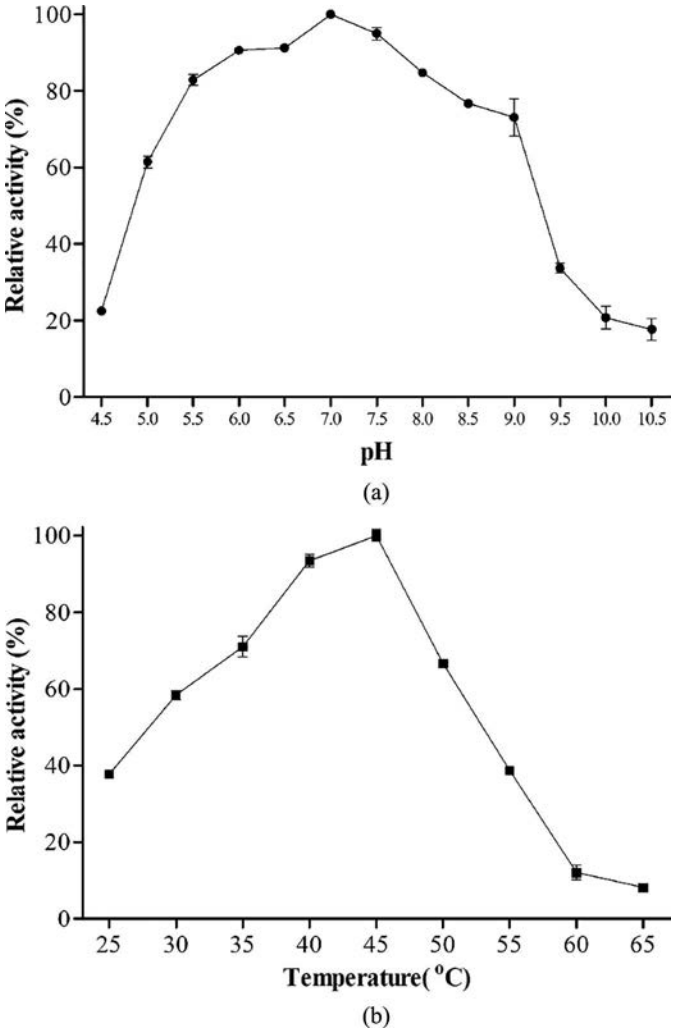

Figure 3. (a) Influence of pH on peptidase activity. The activity was determined in different buffers of varying pH values at 40°C, using azocasein as substrate. (b) Influence of temperature on purified peptidase activity. The activity was determined in 50 m*M* HEPES buffer, pH 7.0, using azocasein as substrate.

Table 2. Effect of inhibitors on peptidase activity from *A. terreus*.

Inhibitor (10 m*M*)	Relative activity (%)
None	100
EDTA	115
IAA	78
PMSF	1

Table 3. Effect of different ions on the activity of serine peptidase from *A. terreus*.

Ions	Residual activity (%)
Control	100 ± 0.7
$MnCl_2$	102.7 ± 2.2
$CaCl_2$	102.6 ± 1,9
$CuCl_2$	89.2 ± 1.3
$BaCl_2$	79.8 ± 3
$MgCl_2$	73.2 ± 0.5
$CoCl_2$	72.3 ± 2.6
KCl	69.2 ± 5.2
NaCl	67.7 ± 2.6
$AlCl_3$	54.3 ± 1.2
LiCl	2.6 ± 0.6

The peptidase activity presented different levels of inhibition in the incubation with 5 m*M* of $CuCl_2$(11%), $BaCl_2$(20%), $MgCl_2$(27%), $CoCl_2$(28%), KCl (31%), NaCl (32%), and $AlCl_3$(46%), and the activity was totality inhibited by LiCl (97%) (Table 3).

Hussain et al.[1] reported that the peptidase of fungus *A. terreus* showed little increase in activity in the presence of Mg^{2+}, Mn^{2+}, and Fe^{3+}. In the conditions used in this study, no ion activates the peptidase, whereas the peptidase of fungi *Aspergillus clavatus* ES1 showed an increase in activity in the presence of calcium and magnesium sulfate.[24] Interestingly, the increase of the activity of the peptidase in the presence of EDTA can be explained by the inactivation observed in the presence of the ions.

Figure 4a shows the effect of the reducing agent DTT on proteolytic activity. The peptidase showed 50% activity when exposed to a range of 20 m*M* to 100 m*M* of DTT; with an increase in concentration of DTT, the activity was maintained at 40%. The peptidase was inhibited by DTT, and this fact indicates that the reducing agent promotes denaturation through the breakdown of the disulfide bonds of the enzyme.[23]

The effect of surfactants SDS and CTAB on enzyme activity showed that the enzyme is sensitive to an increase in surfactant concentration. The enzyme was totally inhibited by 0.1% CTAB and 0.5% SDS (Figure 4b), suggesting that cationic or anionic exchange promotes denaturation. The acid peptidase of *A. clavatus* with 2 m*M* SDS reduces activity by 50%, which indicates breaking of hydrophobic interactions of the native structure.[18] The alkaline serine peptidase produced by *A. clavatus* ES1 was 10% inhibited in 0.1% SDS.[24]

Urea is considered a chaotropic agent and can be associated with the rupture of hydrogen bonds present in enzymes.[25] Figure 4c shows that the peptidase activity was not affected by 1 *M* urea. Merheb-Dinni et al. showed that metallopeptidase increased in activity with an increase of urea concentration until 4.5 *M*, using casein as substrate.[23]

According to the nomenclature described by Schechter and Berger (1967) the catalytic site is divided into subsites (e.g., papain contains seven subsites). Each subsite accommodates an amino acid residue of the substrate. The mapping of subsites reports the size, preference, and other physical and chemical parameters of the active site.[26]

Table 4 shows kinetics of subsites of S_1, S_2, and S_3 of purified enzyme. The study kinetics parameters of the S_1

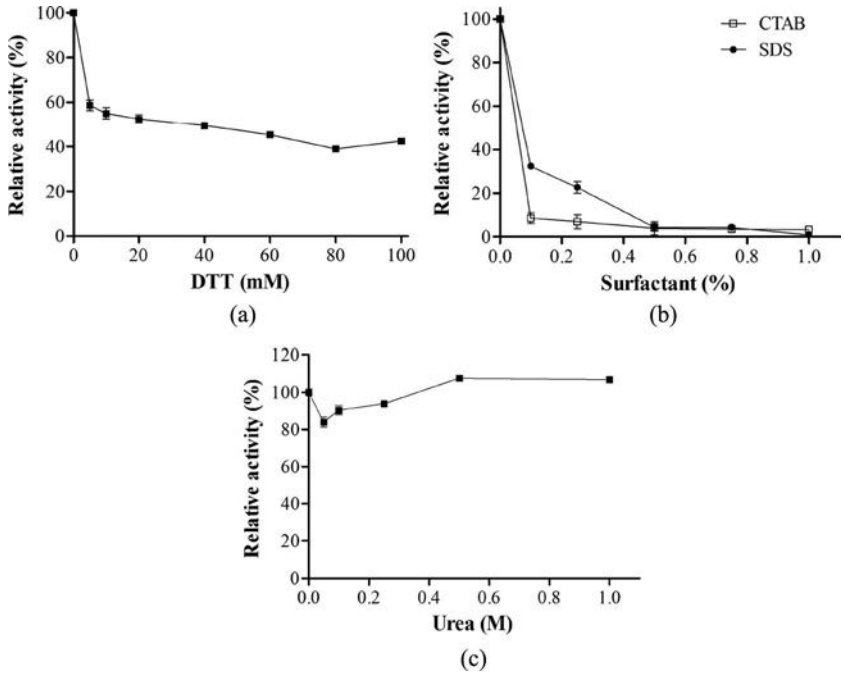

Figure 4. (a) Effect of DTT on peptidase activity. The activity was determined in 50 m*M* Hepes Buffer at 40°C using azocasein as substrate. (b) Effect of cationic (CTAB) and anionic (SDS) surfactants on peptidase activity. The activity was determined in 50 m*M* HEPES buffer at 40°C using azocasein as substrate. CTAB: cetyl trimethyl ammonium bromide; SDS: sodium lauryl sulfate. (c) Effect of urea on peptidase activity. The activity was determined in 50 m*M* HEPES buffer at 40°C using azocasein as substrate.

Table 4. Kinetic parameter (k_{cat}/k_M) for the hydrolysis of the substrates Abz-KLRSSKQ-EDDnp, with replacements in the P_1, P_2, and P_3 position of purified serine peptidase from *A. terreus*.

Substrate P_1 position	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Abz-KLDSSKQ-EDDnp	68.36 ± 0.12
Abz-KLESSKQ-EDDnp	222.7 ± 3.33
Abz-KLFSSKQ-EDDnp	228.9 ± 1.04
Abz-KLHSSKQ-EDDnp	275.2 ± 0.3
Abz-KLISSKQ-EDDnp	Nonhydrolysis
Abz-KLMSSKQ-EDDnp	205.8 ± 1.71
Abz-KLRSSKQ-EDDnp	258.5 ± 0.37
Abz-KLSSSKQ-EDDnp	263.6 ± 2.8
Abz-KLTSSKQ-EDDnp	305.5 ± 0.07
Abz-KLYSSKQ-EDDnp	363.4 ± 2.59
Substrate P_2 position	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Abz-KDRSSKQ-EDDnp	Nonhydrolysis
Abz-KERSSKQ-EDDnp	Nonhydrolysis
Abz-KFRSSKQ-EDDnp	74.7 ± 0.76
Abz-KHRSSKQ-EDDnp	Nonhydrolysis
Abz-KIRSSKQ-EDDnp	98 ± 1.31
Abz-KMRSSKQ-EDDnp	12.4 ± 0.05
Abz-KRRSSKQ-EDDnp	Nonhydrolysis
Abz-KSRSSKQ-EDDnp	Nonhydrolysis
Abz-KLTRSSKQ-EDDnp	Nonhydrolysis
Abz-KLYSSKQ-EDDnp	16.5 ± 0.17
Substrate P_3 position	k_{cat}/K_m ($mM^{-1}\,s^{-1}$)
Abz-DLRSSKQ-EDDnp	201 ± 0.17
Abz-ELRSSKQ-EDDnp	242.7 ± 0.92
Abz-FLRSSKQ-EDDnp	207.3 ± 1.39
Abz-HLRSSKQ-EDDnp	263.6 ± 0.84
Abz-ILRSSKQ-EDDnp	245.7 ± 2.02
Abz-MLRSSKQ-EDDnp	231.7 ± 1.01
Abz-RLRSSKQ-EDDnp	236.4 ± 1.34
Abz-SLRSSKQ-EDDnp	318.7 ± 3.17
Abz-TLRSSKQ-EDDnp	170.7 ± 0.13
Abz-YLRSSKQ-EDDnp	43.7 ± 0.07

subsite showed hydrolysis when the substrate contained amino acid Tyr in this subsite, with a catalytic efficiency of 363.4 ± 2.58 $mM^{-1}\,s^{-1}$, followed by Thr with 305.5 ± 0.07 $mM^{-1}\,s^{-1}$. Subsite S_1 showed a preference for aromatic amino acids in the substrates, which led to the highest degree of hydrolysis. In substrate containing isoleucin the enzyme showed no activity, possibly due to the size of side chain of the amino acid.

Analyzing the peptidase behavior to the S_2 subsite, the peptidase did not show high hydrolysis of the substrates assayed. Only substrates that contained nonpolar amino acids (Ile, Phe, and Met) and Tyr (polar) showed hydrolysis, with catalytic efficiency of 98 ± 2.02, 74.7 ± 0.76, 12.4 ± 0.06, and 16.5 ± 0.17 $mM^{-1}\,s^{-1}$, respectively.

Subsite S_3, displayed highest catalytic efficiency to Ser and His with 318.7 ± 3.17 $mM^{-1}\,s^{-1}$ and 263.6 ± 0.84 $mM^{-1}\,s^{-1}$. This subsite too showed preference for substrates contained polar amino acids. In selectivity order by tested substrates, the S_3 subsite is less selective than the S_1 subsite, and subsite S_2 is more selective than the S_1 and S_3 subsites.

In general, this peptidase showed low catalytic efficiency when compared with the serine peptidase of *Aspergillus fumigatus*,[27] *Penicillium waksmanii*,[16] and *Myceliophtora* spp.[28]

A. terreus	A	L	N	I	E	N	T	V	A	H	G	V	G	Q	I
A. clavatus	A	L	T	T	Q	S	G	A	P	W	G	L	G	S	I

Figure 5. Comparison of N-terminal amino acid sequence of the purified peptidase from *A. terreus* with *A. clavatus* ES1.

The N-terminal sequence of the first 15 amino acid residues of the enzyme isolated from this fungus was ALNIENTVAHGVGQI. Figure 5 demonstrates that the peptidase sequence showed 33% identity with the fungi *A. clavatus* ES1[24] peptidase sequence.

Conclusions

The purification process for the peptidase produced by *Aspergillus terreus* employed in this work was effective and it was performed in three chromatographic steps. The presence of metallic ions had a negative effect on peptidase activity; however, in the presence of EDTA we observed an increase of 15% in its activity. In the kinetics assays, the substrate Abz-KLRSSKQ-EDDnp with replacements in the P_1, P_2, and P_3 positions, respectively, displayed that the subsite S_2 is more specific than subsites S_1 and S_3, but the S_1 subsite showed highest catalytic efficiency. The kinetics parameters showed that the peptidase has a preference for substrates containing polar amino acids in P_1 (Tyr and Trp).

Funding

The authors acknowledge the financial support provided by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (2011/06986-0 and 2012/24703-8) and by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (308078/2012-8).

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