Biochemical and Functional Characterization of a Metalloprotease from the Thermophilic Fungus *Thermoascus aurantiacus*

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Protease production was carried out in solid state fermentation. The enzyme was purified through precipitation with ethanol at 72% followed by chromatographies in columns of Sephadex G75 and Sephacryl S100. It was purified 80-fold and exhibited recovery of total activity of 0.4%. SDS−PAGE analysis indicated an estimated molecular mass of 24.5 kDa and the N-terminal sequence of the first 22 residues was APYSGYQCSMQLCLTCALMNCA. Purified protease was only inhibited by EDTA (96.7%) and stimulated by Fe^{2+} revealing to be a metalloprotease activated by iron. Optimum pH was 5.5, optimum temperature was 75 °C, and it was thermostable at 65 °C for 1 h maintaining more than 70% of original activity. Through enzyme kinetic studies, protease better hydrolyzed casein than azocasein. The screening of fluorescence resonance energy transfer (FRET) peptide series derived from Abz-KL**X**SSKQ-EDDnp revealed that the enzyme exhibited preference for Arg in P_1 (k_{cat}/K_m = 30.1 mM^{-1} s^{-1}).

KEYWORDS: *Thermoascus aurantiacus*; solid state fermentation; metalloprotease; thermostability; fluorescent peptides; N-terminal sequence

INTRODUCTION

Proteases are commercially explored in food, pharmaceutical and chemical industries as biocatalysts with the basic function of hydrolyzing proteins and make up one of the most important groups of industrial enzymes, representing approximately 60% of the total enzyme market (*1*). Thermophilic fungi are characterized by exhibiting minimal growing temperature of 20 °C and maximum growing temperature of 55 °C and generally produce enzymes with some important characteristics such as higher thermostability, optimum activity at elevated temperatures and high hydrolysis rates (*2, 3*). Many researchers have described thermostable protease production by bacteria (*4, 5*); however, the reports of thermostable protease production by thermophilic fungi are few and old (*6−8*).

Thermostable enzymes are usually more robust being more resistant to pH and chemical reagents in the reaction medium. These characteristics are important for the industrial application of enzymes. The use of thermostable enzymes, which promote hydrolysis at high temperatures, is advantageous due to the increase in the speed of reactions and decrease of mesophilic

microbial contamination, improving the technical and economical viability of the process (*9, 3*), ratifying the importance of studying these enzymes.

Biochemical and physicochemical characterization of purified enzymes is important to evaluate their biotechnological potential. Proteases with high specificity such as rennin are important for the dairy industry, in the production of cheeses. Proteases with low specificity are capable of hydrolyzing a larger number of peptide bonds and are important for industrial use in the detergent industry, for example. Thus it is clear that proteases differ in properties such as substrate specificity, optimum catalytic conditions of pH and temperature and stability profiles showing the importance of researches which determine these characteristics for the successful application of these enzymes in their particular industry (*9*).

The thermostable protease from the thermophilic fungus *Thermoascus aurantiacus* was reported for the first time in the crude extract in a previous work in our laboratory (*3*). The present work has the aim of studying the purification and characterization of this protease obtained from solid state fermentation using wheat bran and casein as substrate. We also investigated the specificity of protease subsite S_1 using fluorescence resonance energy transfer (FRET) peptides derived from

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Article

J. Agric. Food Chem., Vol. 57, No. 19, 2009 **9211**

the peptide Abz-KLXSSKQ-EDDnp (Abz = *o*-aminobenzoic acid; EDDnp = *N*-[2,4 dinitrophenyl]ethylenediamine; Abz/EDDnp = donor/acceptor fluorescent pair) as a means of learning more about enzyme specificity.

MATERIALS AND METHODS

Microorganism and Inoculum. The fungus *Thermoascus aurantiacus*, obtained from Laboratory of Biochemistry and Applied Microbiology–IBILCE–UNESP, was maintained in slants of Sabouraud dextrose agar medium (Oxoid) at room temperature. Before each experiment, the organism was transferred to 250 mL Erlenmeyer flasks containing 50 mL of Sabouraud dextrose agar medium (Oxoid) that were then incubated at 50 °C for two days until complete growth. To each flask 100 mL of sterilized nutrient solution made up of 0.1% (w/v) (NH$_4$)$_2$SO$_4$, MgSO$_4$·7H$_2$O and NH$_4$NO$_3$ was added and the mycelium suspended. This suspension was used to inoculate the culture mediums.

Solid-State Fermentation and Enzyme Extraction. Media containing wheat bran and 5% of casein, totaling 5 g of substrate, were sterilized (120 °C/20 min) in 250 mL Erlenmeyer flasks. These were inoculated with 10.5 mL of mycelial suspension, to approximately 70% moisture, and incubated at 45 °C for 48 h. The crude enzyme solution was obtained by adding 40 mL of distilled water to the fermented material followed by agitation on rotary shaker at 100 rpm/20 min. Solids were removed by filtering and centrifuging at 10000*g*/20 min at 5 °C, and the clear solution was used.

Proteolytic Activity. *Casein Method.* The casein method was according to Kembhavi et al. (*10*), with modification. The reaction mixture was made up of 0.5 mL of casein (Sigma) 1% (w/v) in 0.1 M acetate buffer, pH 5.5 to which 0.2 mL of the enzyme solution was added. The reaction was carried out at 55 °C and stopped after 20 min with 0.6 mL of TCA (trichloroacetic acid) 10%. Test tubes were centrifuged at 10000*g*/10 min at room temperature, and the absorbance of the supernatant was measured at 280 nm. An appropriate control was prepared in which TCA was added before the enzymatic solution. One unit of enzyme activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 280 nm, under the assay conditions.

Azocasein Method. The azocasein method was according to Germano et al. (*11*), with modification. The reaction mixture was made up of 0.3 mL of azocasein (Sigma) 1% (w/v) and 0.15 mL of 0.5 M MES buffer, pH 5.5, to which 0.15 mL of enzyme solution was added. The reaction was carried out at 55 °C and stopped after 20 min with the addition of 1.2 mL of TCA 10%. Test tubes were centrifuged at 10000*g*/20 min at room temperature, 1.2 mL of the supernatant was neutralized with 1.4 mL of 1 M NaOH and, after agitation, the absorbance was measured at 440 nm. An appropriate control was prepared in which TCA was added before the enzymatic solution. One unit of enzyme activity (U) was arbitrarily defined as the amount of enzyme required to cause an increase of 0.1 in absorbance at 440 nm, under the assay conditions.

Specific activity was expressed as units of enzymatic activity per mg of protein.

Protein Content. Protein content was measured, following the method of Hartree (*12*), using bovine serum albumin (BSA) as standard.

Protease Purification. *Concentration of Crude Enzymatic Extract.* The crude enzymatic extract was first subjected to precipitation with commercial alcohol (96%) at 72% (v/v). After this solution was left to rest for 12 h at 5 °C it was centrifuged at 13000*g*/20 min at 1 °C, and the pellet obtained was suspended in a minimal volume of 50 mM acetate buffer, pH 5.5.

Gel Filtration Chromatographies. The concentrated enzyme was subjected to gel filtration on a Sephadex G75 column (100 cm × 3 cm) equilibrated with 50 mM acetate buffer, pH 5.5 containing 50 mM NaCl. Fractions of 3.5 mL were collected at a flow rate of 0.3 mL/min with the same buffer. Protein content (280 nm) and protease activity were measured.

Fractions showing protease activity were pooled and concentrated in Centriprep (Millipore) with cutoff of 10 kDa and submitted to a second gel filtration on a Sephacryl S100 (65 cm × 2 cm) equilibrated with 50 mM acetate buffer, pH 5.5 containing 0.2 M NaCl. Enzyme fractions of 3.5 mL were collected at a flow rate of 0.3 mL/min with the same buffer.

SDS–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out for the determination of purity of the enzyme as described by Laemmli (*13*). Proteins were denaturized in boiling water bath for 5 min in electrophoresis sample buffer containing 0.1 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT and 0.001 M bromophenol blue. Twelve percent running gel and 4% stacking gel were prepared according to Sambrook and Russel (*14*). Staining was performed according to Blum et al. (*15*).

Characterization of Purified Enzyme. *Effect of pH and Temperature on the Activity and Thermal Stability of the Enzyme.* Optimum pH was determined by assaying proteolytic activity at 55 °C at different pH values, using the following 50 mM buffer solutions: acetate (pH 3.5–5.0); Mes (pH 5.5–7.0); Taps (pH 7.5–9.0) and Caps (9.5–11.5). Optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 35 to 85 °C and assaying the activity at the pH determined as optimum.

Thermal stability was examined by incubating the enzyme at temperatures ranging from 60 to 95 °C for 270 min. Aliquots were taken at desired time intervals to test the remaining activity at pH and temperature determined as optimum. The nonheated enzyme was considered as a control (100%).

Effect of NaCl, Surfactants, Metal Ions and Inhibitors on Proteolytic Activity. The effect of UREA and DTT on proteolytic activity was investigated using different concentrations of urea (0 to 5 M) and DTT (0 to 0.3 M). Samples were incubated at room temperature for 5 min, and then 50 mM Mes buffer, pH 5.5 and casein 1% were added and the residual activity was determined and compared to the control, which was incubated without the agents and corresponds to 100%.

The effect of NaCl on proteolytic activity was investigated using azocasein or casein 1% in the reaction mixture with different concentrations of the salt (0; 0.2; 0.4; 0.6; 0.8; 1.0; 1.5; 2.0; 2.5 and 3.0%). The effects of some surfactants (Triton X-100, Tween-20) on proteolytic activity were studied using casein in the reaction mixture with different concentrations of the surfactants (0.1; 0.2; 0.4; 0.6; 0.8; 1.0%). Proteolytic activity was determined and compared to the control, which was incubated without these agents and corresponds to 100%.

The effect of various metal ions (10 mM) on proteolytic activity was studied using different salts including MnCl$_2$, NH$_3$Cl, MgCl$_2$, KCl, BaCl$_2$, HgCl$_2$, CaCl$_2$, CuCl$_2$, CaSO$_4$, FeSO$_4$, NH$_3$SO$_4$, CuSO$_4$, K$_2$SO$_4$, MnSO$_4$ and MgSO$_4$. Samples were incubated at room temperature for 5 min, and then 50 mM Mes buffer, pH 5.5 and casein 1% were added and the residual activity was determined and compared to the control, which was incubated without the ions and corresponds to 100%.

In order to classify the protease, different specific inhibitors (10 mM) were added to the enzyme solution including serine protease inhibitor (phenylmethylsulfonyl fluoride, PMSF), metalloprotease inhibitor (ethylenediamine tetraacetic acid, EDTA), cysteine protease inhibitor (iodoacetic acid). Samples were incubated at room temperature for 5 min, and then 50 mM Mes buffer, pH 5.5 and casein 1% were added and the residual activity was determined and compared to the control, which was incubated without the inhibitors and corresponds to 100%.

Enzyme Kinetics on Casein and Azocasein Substrates. For the assay of kinetic parameters the substrate concentration was varied between 0.25 and 6.0 mg/mL and between 0.125 and 6.0 mg/mL for casein and azocasein, respectively. Kinetic constants (K_m, V_{max}) were determined at 8.2 μmol concentration of the purified protease through nonlinear regression using GraFit version 5.0 (*16*). The catalytic constant (k_{cat}) was determined as follows: $k_{cat} = V_{max}/[E] \times 1000/60$.

Fluorescence Resonance Energy Transfer (FRET) Substrates. With the purpose of determining enzyme specificity, we used internally quenched fluorescence peptides with substitutions in P$_1$ from the series Abz-KLXSSKQ-EDDnp.

Peptides were synthesized by the solid-phase synthesis method as described elsewhere (*17*) in an automated solid-phase peptide synthesizer, Shimadzu Model PSSM-8. The series of peptides Abz-KLXSSKQ-EDDnp were purified by semipreparative HPLC, and their molecular weight and purity were checked by amino acid analysis and by molecular mass determination with MALDI-TOF mass spectrometry, using a TofSpecE from Micromass. The stock solutions of these peptides were prepared in DMSO, and the concentrations were measured on a Cary

Table 1. Summary of the Purification of Protease F3 from *T. aurantiacus*

purification steps	total activity (U)	total protein (mg)	sp act. (U/mg)	recovery (%)	purification (fold)
crude extract	13288.00	457.60	29.04	00	1
precipitation	7321.56	76.15	96.15	55.1	3.30
F III Sephadex G75	1593.90	7.70	207.00	12.0	7.13
Sephacryl S100	46.20	0.02	2310.00	0.4	79.5

100 Scan spectrophotometer using the molar extinction coefficient of 17,300 M^{-1} cm^{-1} at 365 nm.

Kinetic Assays on Synthetic Substrates. Michaelis−Menten kinetic parameters were determined by initial velocity measurements at varying substrate concentrations. The hydrolysis of the fluorogenic peptide substrates was assayed using a RF-5301PC spectrofluorimeter (Shimadzu Corporation). Using this approach we were able to calculate the kinetic parameters (k_{cat}, K_m, k_{cat}/K_m) for the hydrolysis of 17 substrates with substitutions at **X** by various amino acids, trying to determine the preference of subsite S_1 (*18*) for peptide cleavage. The assays were performed at 55 °C in 50 mM acetate buffer, pH 5.5. The fluorescence was measured continuously at λ_{ex} = 320 nm and λ_{em} = 420 nm (1.54−1.57 mL final sample volume). The concentration of the active enzyme (0.58 μmol) was determined by active site titration with EDTA (*19*). The cuvette's path length was 1 cm, and the enzyme was preincubated for 3 min before adding the substrate. The slope was converted into micromoles of substrate hydrolyzed per min based on a calibration curve obtained from the complete hydrolysis of each peptide (*20*). The kinetic parameters V_{max} and K_m were calculated by nonlinear regression data analysis using Grafit version 5.0 (*16*), allowing the calculation of k_{cat} and the catalytic efficiency k_{cat}/K_m. The errors were less than 5% for any obtained kinetic parameter value.

N-Terminal Amino Acid Sequencing. The N-terminal sequence of the purified protease was determined using the Protein Sequencer PPSQ-33A (Shimadzu Corporation, Kyoto, Japan), which sequentially cleaves the N-terminal amino acids using Edman degradation.

RESULTS AND DISCUSSION

Protease Purification from the Crude Enzymatic Extract. For the purification studies the enzyme produced with wheat bran supplemented with 5% of casein was chosen since it exhibited proteolytic activity on the second day of fermentation similar to the one supplemented with 20% of casein (results not shown). The advantage is to have less protein in the crude extract interfering on the purification procedures.

Purification of one of the extracellular proteases from *T. aurantiacus* was carried out by a combination of conventional purification procedures.

In the first step, the crude enzymatic extract was precipitated with alcohol. The 72% (v/v) alcohol fraction showed a recovery of approximately 80% and purification fold of approximately 3. The method of precipitation using organic solvents was also used for other fungal proteases (*21*, *22*). This fraction was then subjected to gel filtration on a Sephadex G75 column, which separated the concentrated enzyme solution in 3 fractions with proteolytic activity (**Figure 1A**).

Fraction III was chosen for further purification employing gel filtration on Sephacryl S100 since it exhibited higher proteolytic activity and lower protein content, as seen in **Figure 1B**. The elution profile from Sephacryl S100 is shown in **Figure 2A**. The presence of only one peak with proteolytic activity can be clearly visualized.

Purified protease subjected to SDS−PAGE yielded a single band (**Figure 2B**) suggesting that this protein is a monomer, and its molecular mass was estimated to be 24.5 kDa.

The results of the purification procedures are summarized in **Table 1**. After the final purification step, the enzyme was purified 80-fold with a recovery of 0.4% and a specific activity of 2310.0 U/mg of protein. The low recovery may be explained by
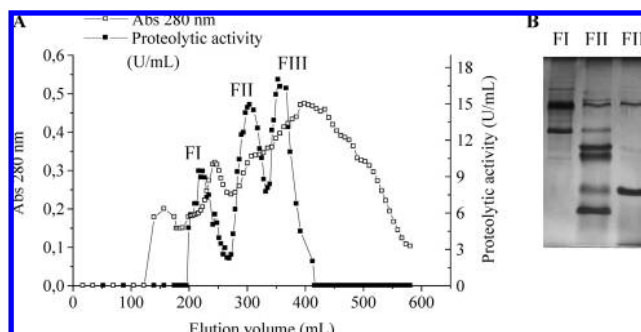

Figure 1. (**A**) Elution profile of protease by gel filtration on Sephadex G75 column. (**B**) SDS−PAGE 12% of fractions eluted from gel filtration on Sephadex G75 column.

the initial separation of total proteolytic activity in 3 different fractions with proteolytic activity. This implied a lower enzyme quantity for the next chromatographic step.

There are reports of protease purification through the use of traditional procedures that involve precipitation and chromatographic techniques, and the low molecular mass exhibited by the protease is in agreement with other research in which fungal proteases are generally lower than 50 kDa (*23*, *21*, *7*). Differently from other authors, the purification protocol we achieved did not employ ion exchange chromatography. This was possible since the fractions exhibited very distinct molecular masses, allowing separation in gel filtration processes.

Characterization of Purified Protease. The effect of pH on the hydrolysis of casein and azocasein by protease F3 was determined over the range 5.5−11.5. Protease F3 showed a significant variation of enzyme activity with pH change, showing two maximum points at 5.5 and 10.5 when using casein and 5.5 when using azocasein (**Figure 3**). In spite of the fact that most metalloproteases exhibit optimum pH near neutrality (*9*) protease F3 from *T. aurantiascus* exhibited a different behavior since its optimum pH was approximately 5.5. A similar result was reported by Hatanaka et al. (*24*), which studied a metalloprotease SSMP, produced by *Streptomyces septatus* TH-2, exhibiting optimum pH of 6.0.

The difference in optimum pHs when using the different substrates may be explained by the fact that these substrates are proteins and also that they exhibit states of protonation and deprotonation, which may alter the bonds between the substrate and the catalytic site and consequently affect catalysis.

The temperature effect under the tested experimental conditions showed a maximum at 75 °C when using casein as substrate (**Figure 4**). The ability to act at elevated temperatures is a characteristic exhibited by enzymes from thermophilic microorganisms. This is of great biotechnological importance for allowing processes to occur at high temperatures, which reduces the risk of contamination by mesophilic microorganisms, favors substrate and product solubility and increases reaction rates for reducing viscosity and for increasing the diffusion coefficients of substrates (*2*).

Other researches (*22*, *23*) show that optimum temperatures for fungal proteases vary from 40 to 60 °C. However, proteases from
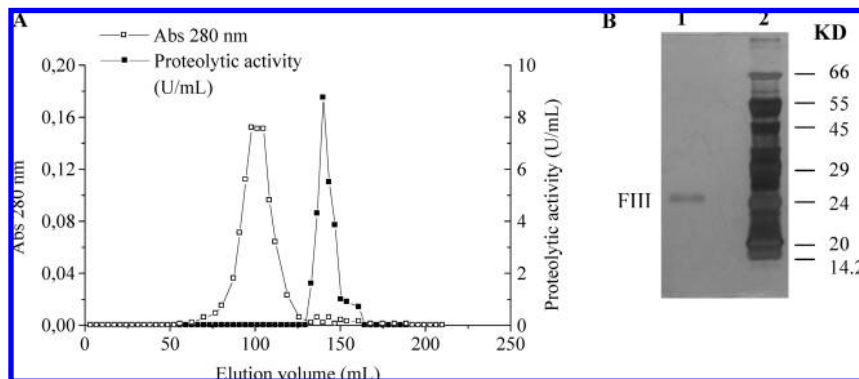
Figure 2. (**A**) Elution profile of protease by gel filtration on Sephacryl S100 column. (**B**) SDS−PAGE 12% of the purified protease from *T. aurantiacus*. Lane 1, purified protease after gel filtration on Sephacryl S100, and lane 2, molecular mass markers: BSA, 66 kDa; albumin, 55 kDa; ovoalbumin, 45 kDa; glyceraldehyde-3-P desydrogenase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; α-lactoalbumin, 14.2 kDa.
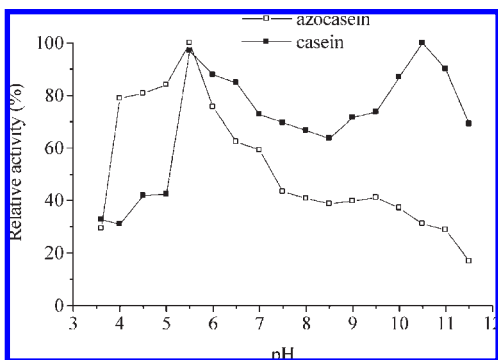

Figure 3. Effect of pH on the proteolytic activity of protease F3. Assay performed at 55 °C.
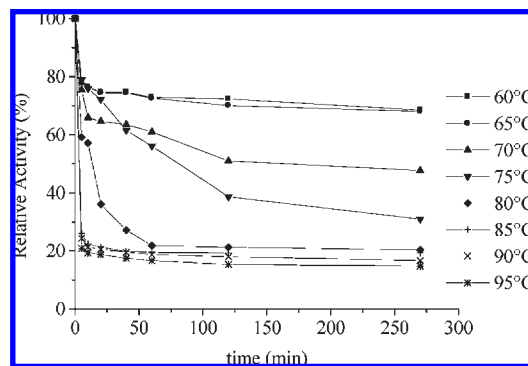

Figure 5. Denaturation temperature of protease F3 using casein as substrate. Assay performed at different temperatures for different times (5, 10, 20, 40, 60, 120, and 270 min).
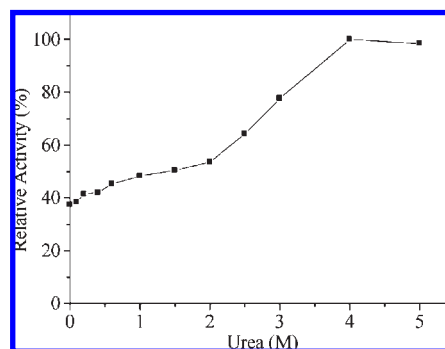

Figure 4. Temperature effect on the proteolytic activity of protease F3 using casein as substrate. Assay performed at pH 5.5.

Figure 6. Proteolytic activity of protease F3 in the presence of urea using casein as substrate. Assay performed at pH 5.5 and 55 °C.

thermophilic fungi show higher values for optimum temperature such as 70 and 75 °C, as shown in the studies of Li et al. (*6*) with *Thermomyces lanuginosus* and Hashimoto et al. (*7*) with *Penicillium duponti* K1014, respectively, which agrees with our results.

The protease F3 was shown to be resistant to denaturation temperatures (**Figure 5**). It maintained 70% of catalytic activity after 270 min at 60 and 65 °C; when exposed to 70 °C for 270 min it maintained 50% of catalytic activity, and at 75 °C for 120 min it maintained 30% of activity. At temperatures of 80 up to 95 °C protease F3 did not exhibit satisfactory catalytic activity, with 15 to 25% of activity after 270 min of incubation.

The high thermostability of protease F3 agrees with reports of other enzymes secreted by *Thermoascus aurantiacus* such as oxidase, 100% stability at 60 °C for 15 min (*25*); endoglucanase,

100% stability at 50 °C for 48 h (*26*); xylanase, 100% stability at 60 °C for 1 h (*27*).

Similar results of high thermostability are generally found for proteases produced by bacteria such as *Bacillus* sp. JB-99 (*5*) with 63% of activity at 70 °C, *Bacillus licheniformis* NH1 (*4*) which maintained 100% of activity at 60 °C in the presence of CaCl₂, *Bacillus stearothermophilus* TLS33 (*28*) which exhibited a little over 60% of activity at 70 °C, *Bacillus* sp. SMIA-2 (*29*) which produced a protease with 80% of activity at 70 °C in the crude extract. Other thermophilic fungi such as *Thermomyces lanuginosus* (*6*) and *Malbranchea pulchella* var. *sulfurea* (*8*) also produce proteases with high thermostability: approximately 70% of activity at 60 °C for the enzyme in the crude extract and 50% of activity at 73 °C, respectively.
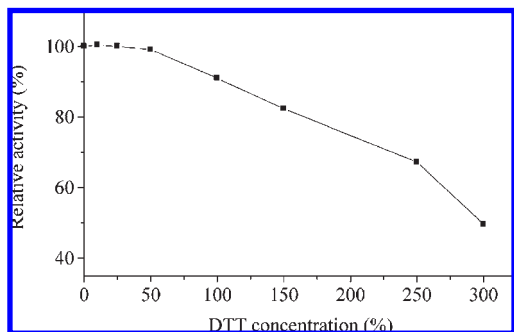
Figure 7. Proteolytic activity of protease F3 in the presence of DTT using casein as substrate. Assay performed at pH 5.5 and 55 °C.
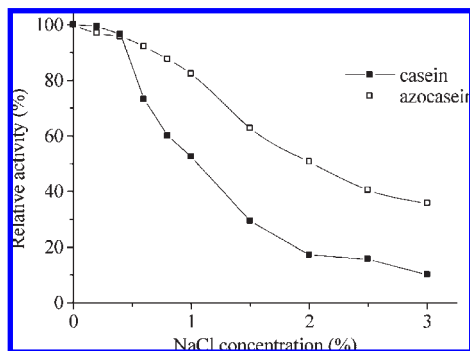

Figure 9. Proteolytic activity of protease F3 in the presence of surfactants using casein as substrate. Assay performed at pH 5.5 and 55 °C.

Table 2. Effect of Different Protease Inhibitors on the Activity of Protease F3

inhibitor (10 mM)	residual activity (%)
none	100
PMSF	97
IAA	96
EDTA	3

Figure 8. Proteolytic activity of protease F3 in presence of NaCl. Assay performed at pH 5.5 and 55 °C.

Figure 6 shows the proteolytic activity in the presence of varying concentrations of urea when using casein as substrate. Chaotropic agents such as urea disrupt protein structure by interfering with hydrogen bonds (*30*). We can observe that, in casein, proteolysis increases with 4.0 M urea, decreasing smoothly at higher concentrations, reaching an activity equal to the control at the highest tested concentration (5 M). The denaturation of casein, which could imply better enzyme access, and an increase of the stability of the enzyme when bound to the substrate could account for the increased hydrolysis up to 4.0 M urea. Casein may have displayed a protective effect over protease F3, avoiding enzyme denaturation in the presence of urea.

Figure 7 shows the effect of DTT on the proteolytic activity of protease F3. DTT is a reducing agent that causes the breakdown of disulfide bonds formed between sulfhydryl groups. This bond is of great importance for stabilizing the tertiary structure of proteins, along with hydrophobic interactions (*31*). Protease F3 was stable at a concentration of 50 mM DTT, and above it proteolytic activity started to decrease. With 300 mM, protease F3 exhibited 50% of its original catalytic activity. The loss of proteolytic activity may be related to the breakdown of disulfide bonds, causing denaturation of the tertiary structure of the enzyme. According to Sumantha et al. (*9*), the protease extracted from *Aspergillus oryzae* NRRL 2217 exhibited complete loss of catalytic activity in the presence of 100 mM DTT. Thus, protease F3 from *T. aurantiascus* revealed to be more resistant than protease from *Aspergillus* sp., since it exhibited 90% of catalytic activity in the same concentration of DTT and with 300 mM exhibited a loss of 50% of activity.

Figure 8 shows the effect of NaCl on the proteolytic activity of the protease F3, using casein and azocasein as substrate. It can be seen that NaCl had an influence over catalytic activity since an increase of NaCl concentration led to a decrease of activity, which could be due to the neutralization of charges between substrate
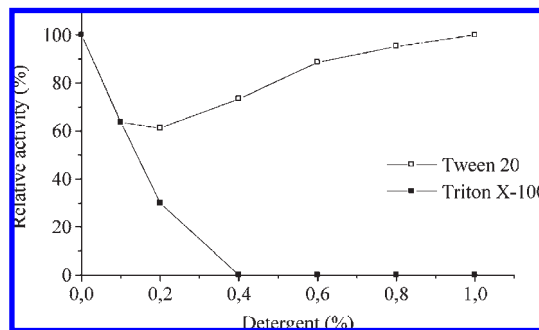
and enzyme, hindering the bond between substrate and active site. When using azocasein as substrate, protease F3 was shown to be more resistant to the increase of NaCl concentration probably because it is a synthetic substrate.

Enzymes are usually inactivated in the presence of surfactants in the reaction medium (*32*). **Figure 9** shows the effect of nonionic surfactants on proteolytic activity, and it can be seen that the surfactants tested presented different effects. In the presence of Triton X-100 protease F3 suffered loss of activity as the concentration of the surfactant increased and with 0.4% protease was no longer active. Differently, surfactant Tween-20 presented a less damaging effect over catalytic activity since with 0.4% protease it exhibited 70% of original activity and with 1% it recovered 100% of its activity. Similar behavior regarding the presence of surfactants, decrease of activity by Triton X-100 and increase of activity by Tween-20, was also reported for the protease from *Clonostachys rosea* (*33*), which exhibited 36.5% of inhibition by Triton X-100 and 16% of activation by Tween-20.

Table 2 shows the effect of different protease inhibitors on proteolytic activity, in order to determine the nature of protease F3. Due to the inhibition of proteolytic activity in the presence of EDTA and the maintenance of activity in the presence of serine and cysteine protease inhibitors, it can be concluded that this is a metalloprotease.

Sumantha et al. (*9*) also reported the existence of a metalloprotease produced by *Aspergillus oryzae* NRRL 2217. However, this behavior is generally seen for proteases from bacteria such as *Bacillus stearothermophilus* TLS33 (*28*), *Serratia marcescens* (*34*) and *Chryseobacterium taeanense* TKU001 (*32*).

Table 3 shows the effect of ions over catalytic activity of protease F3. It can be seen that protease F3 was inhibited by the presence of mercury chloride and exhibited approximately 50% and 43% of activity loss in the presence of copper sulfate and copper chloride, respectively, showing that copper has a negative effect over protease F3. Similar results were reported by Nascimento et al. (*29*) for the protease obtained from *Bacillus* sp. Protease F3 was activated by Fe^{2+}, which was also reported for protease from *Aspergillus oryzae* NRRL 2217 (*9*) and for metalloprotease from *Bacillus subtilis* FP-133 (*35*). The effect of ions over protein stability may be caused through chemical interactions between them, with the formation of complexes, such as ions
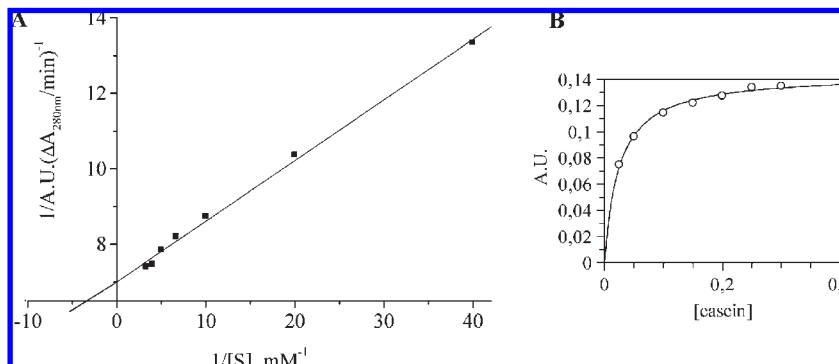
Figure 10. Effect of substrate concentration on the reaction velocity: Lineweaver–Burk plot of initial velocity of reaction on casein (**A**) and Michaelis–Menten curve (**B**), where A.U. = $\Delta Abs_{280nm} \times 10/20$ min.
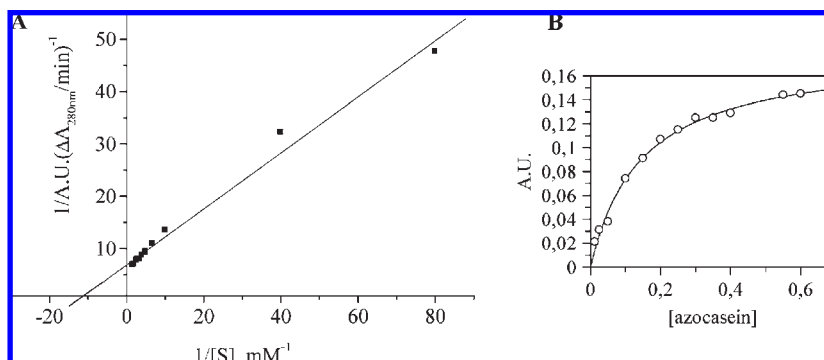

Figure 11. Effect of substrate concentration on the reaction velocity: Lineweaver–Burk plot of initial velocity of reaction on azocasein (**A**) and Michaelis–Menten curve (**B**), where A.U. = $\Delta Abs_{440nm} \times 10/20$ min.

Table 3. Effect of Different Ions on the Activity of Protease F3

ions	residual activity (%)
control	100.00
$MnCl_2$	86.67
NH_3Cl	89.41
$MgCl_2$	92.94
KCl	89.02
$BaCl_2$	94.12
$HgCl_2$	5.49
$CaCl_2$	89.80
$CuCl_2$	57.65
$CaSO_4$	96.86
$FeSO_4$	197.25
NH_3SO_4	96.86
$MnSO_4$	100.78
$CuSO_4$	51.76
K_2SO_4	90.20
$MgSO_4$	94.90

Table 4. Kinetic Parameters of Protease F3 Using Casein and Azocasein as Substrates[a]

substrate	V_{max} (mM/min)	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
casein	0.1443	0.0243	12.07
azocasein	0.1801	0.1446	2.53

[a] Results obtained through nonlinear regression using GraFit 5.0 program. The errors were kept below 10%.

used as substrates, cosubstrates or cofactors. The effect of ions over enzyme activity may be caused by competitive or noncompetitive inhibition of the enzyme (*36*). According to Zhao (*36*) halophilic enzymes, such as menadione redutase from *Halobacterium cutiru-brum*, require high salt concentrations to keep their activity and stability high (2 to 3 M of NaCl for optimum activity). Therefore, observing the results, some of the salts studied exhibited an activating effect on the enzyme while others exhibited an inhibiting effect, which were probably caused by the chemical interactions between enzyme, substrate and salts. Protease F3 is a metalloprotease, and this class of proteases is characterized by its requirement of divalent metal ions for its catalytic activity (*37*), which corroborates with the importance of Fe^{2+} for enzymatic activity. Metalloproteases are usually associated with Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}; therefore protease F3 is a different metalloprotease.

The results from the enzymatic kinetics studies are presented in **Table 4** and in **Figures 10** and **11**. When using casein as substrate, values for K_m of 0.0243 mM, for V_{max} of 0.1443 mM/min and for specificity constant (k_{cat}/K_m) of 12.07 mM^{-1} s^{-1} were obtained. When using azocasein, values for K_m of 0.1446 mM, V_{max} of 0.1801 mM/min and k_{cat}/K_m of 2.53 mM^{-1} s^{-1} were found (**Table 4**).

The higher value of K_m and the lowest value of k_{cat}/K_m for the reaction with azocasein indicate less affinity of the enzyme for this substrate, when compared to casein, meaning that the enzyme requires lower concentrations of casein to achieve maximum catalytic efficiency. This might also have happened because the protease acts with low specificity, and the casein molecule contains more cleavage sites. When using azocasein, activity is quantified based on the hydrolysis of the bond between chromophore and casein.

In the studies of Chakrabarti et al. (*21*) protease from *A. terreus* (IJIRA 6.2) exhibited a K_m value for its reaction with casein of 0.055 mM, being slightly less efficient for this reaction than protease from *T. aurantiacus*.

Table 5 and **Figure 12** display the data gathered concerning the specificity of the S_1 subsite. It was probed with the peptide series

Table 5. Kinetic Parameters for the Hydrolysis of the FRET Peptide Series Abz-KL**X**SSKQ-EDDnp (P[1]) by Protease F3 for the Characterization of Its S[1] Specificity[a]

X	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (mM s)$^{-1}$
R	0.52	17.3	30.1
K	0.19	13.0	14.9
C	0.15	11.9	12.7
F	0.09	14.6	6.4
M	0.06	9.5	6.1
Y	0.06	11.3	5.6
Q	0.03	8.6	3.4
H	0.05	14.4	3.2
V	0.03	13.5	2.0
W	0.01	5.7	1.5
E	0.01	9.2	0.6
T	0.01	11.0	0.4
A		b	
N		b	
G		b	
P		b	
I		b	

[a] Assay performed at pH 5.5 and 55° C. [b] No significant hydrolysis was detected.
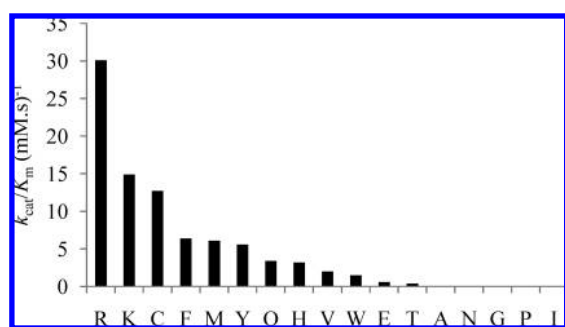
Figure 12. k_{cat}/K_m values for hydrolysis by protease F3 of the FRET peptide series Abz-KL**X**SSKQ-EDDnp (P[1]). Experimental conditions: pH 5.5, 50 mM acetate buffer at 55 °C.

Abz-KLXSSKQ-EDDnp (where **X** indicates Arg, Ile, Lys, Cys, Phe, Met, Tyr, Gln, His, Val, Trp, Asp, Thr, Ala, Asn, Gly and Pro). The enzyme displayed the highest catalytic efficiency with Arg. For other two residues the protease showed significant efficiency too, but at lower level than that for the formerly mentioned amino acid: Lys and Cys, in decreasing order. Their side chains possess distinct physical and chemical properties. Considering Lys, it belongs to the basic side chain amino acids with an aliphatic side chain and a positively charged group at its terminus such as Arg, while Cys is a polar uncharged residue. Thus, the specificity of the metalloprotease from *Thermoascus aurantiacus* differs from that of metalloprotease from *Serratia proteamaculans* which exhibited preference for Gly, a nonpolar amino acid, for S[1] subsite (*38*). The amino acids with aromatic side chains were poor substrates, and among those residues with undetected hydrolysis, we find Pro, which frequently displays this behavior due to its side chain carrying an imide bond. Based on these results, we suggest that the protease prefers polar amino acids in P[1], followed by nonpolar residues. However, a more complete analysis comprising studies with LC−MS or MALDI-TOF mass spectrometry is necessary to confirm the cleavage site by the protease from *T. aurantiacus* fungus.

The N-terminal sequence of the first 22 residues was APYSGYQCSMQLCLTCALMNCA, and it did not reveal homology to any known proteins from the BLAST network service (*39*), except for protease from *Cryptococcus neoformans* var. *neoformans* JEC21 (*40*) which exhibited similarity only to residues 12 to 21, suggesting that this might be a new protein.

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