Abstract  An extracellular alkaline serine protease has been purified from a strain of *Aspergillus clavatus*, to apparent homogeneity, by ammonium sulfate precipitation and chromatography on Sephadex G-75. Its molar mass, estimated by SDS-PAGE, was 35 kDa. Maximum protease activity was observed at pH 9.5 and 40°C. The enzyme was active between pH 6.0 and 11.0 and was found to be unstable up to 50°C. Calcium at 5 mM increased its thermal stability. The protease was strongly inhibited by PMSF and chymostatin as well as by SDS, Tween 80 and carbonate ion. Substrate specificity was observed with *N*-p-*Tos*-Gly-Pro-Arg-*p*-nitroanilide and *N*-Suc-Ala-Ala-Ala-*p*-nitroanilide being active substrates. Parts of the amino acid sequence were up to 81% homologous with those of several fungal alkaline serine proteases.

Introduction

The alkaline serine proteases (EC 3.4.21), active and stable in the alkaline pH, are the most important group of commercial enzymes and are utilized in the detergent, food, pharmaceutical and leather industries and silver recovery and play an important role in the cellular metabolic processes (Godfrey and West 1996).

Alkaline proteases from *Aspergillus* species are used in leather treatment, endo- and exoproteases from *A. oryzae* have been used to modify wheat gluten, an insoluble protein, by limited proteolysis facilitating its handling and machining and these proteases are also used in the health treatments (Chiplonkar et al. 1985). Recently, we reported the best conditions for the alkaline protease production by this strain (Tremacoldi and Carmona 2005). The aim of this work was to purify and biochemically characterize the extracellular alkaline protease from this isolate after cultivation under optimized conditions.

Material and methods

Microorganism and growth

*Aspergillus clavatus* strain CCT2759 from the culture collection of Fundação Tropical de Pesquisas e Tecnologia André Tosello, Campinas, Brazil, was isolated from soil of the Atlantic forest, Brazil. This strain was selected from many fungal strains as the best extracellular protease producer (Attili 1994).

In the lab, it was cultivated on Vogel solid medium (Vogel 1956) at 25°C, in the absence of light. A suspension (1 ml) containing 10⁷ spores/ml, from...
7-day-old colonies, was inoculated into Erlenmeyer flasks (250 ml) with 50 ml Vogel medium (pH adjusted to 7) with 1% (w/v) glucose as carbon source and 1% casein as nitrogen source. The suspension was incubated for 4 days at 25°C in a shaker at 120 rpm. The mycelium was removed by vacuum filtration and the filtrate was used as enzyme source.

Enzyme assay

Alkaline proteolytic activity was determined at 37°C for 90 min using 2% casein (w/v) in 100 mM glycine NaOH buffer pH 9.5. The reaction was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid, followed by centrifugation at 6000 × g for 20 min at 4°C and the absorbance of the supernatant was determined at 280 nm. One unit of enzyme activity (U) was defined as the enzyme amount catalysing an increase of 0.1 in the absorbance at 280 nm, per ml of sample during 1 h of incubation (Kundu et al. 2000). Protein concentration of each sample was estimated by the Bradford method with modification (Sedmack and Grossberg 1977) with bovine serum albumin as standard.

Purification of protease

Filtrate proteins were fractionated by ammonium sulfate precipitation (40–75%, w/v). The precipitate was recovered by centrifugation (6000 × g) for 20 min at 4°C and the absorbance of the supernatant was determined at 280 nm. The precipitate was dissolved in 9.6 ml of 50 mM ammonium acetate buffer pH 6.8 and dialysed overnight against 50 volumes of this buffer. This sample was chromatographed on Sephadex G-75 (1.8 × 60.5 cm column) equilibrated with the same buffer. Elution was done at a flow rate of 18.0 ml h⁻¹ and 3.0 ml fractions were collected. Absorbance at 280 nm was measured and proteolytic activity was determined. The fractions exhibiting significant activity were pooled and the obtained sample was submitted to electrophoresis. SDS-PAGE 10% electrophoresis was done according to Laemmli (1970). Proteins were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 diluted in methanol–acetic acid–water (5:2:5, v/v/v). All the steps were carried out at 4°C.

Effects of temperature and pH on activity and stability

Optimum temperature was determined by activity assay at 20–65°C. Thermal stability was investigated by preincubation of the enzyme without substrate at 40, 45, 50 and 55°C from 0 to 180 min, before activity assay. To test the influence of Ca²⁺ on proteolytic activity, 50 mM CaCl₂ was added to the sample during preincubation at 50, 55 and 60°C. Optimum pH was determined using McIlvaine (pH 3–8), 100 mM glycine NaOH (pH 9–10.5) and 100 mM sodium phosphate (pH 11–12) buffers at 40°C. The pH stability was verified by incubation of the enzyme for 24 h, in those buffers at 4°C, before the enzymatic activity assay.

Hydrolysis of chromogenic substrates

Chromogenic substrates used were α-benzoyl-d-Arg-p-nitroanilide, N-succinyl-Ala-Ala-Val-p-nitroanilide, N-succinyl-L-Phe-p-nitroanilide acetate and N-p-tosyl Gly-Pro-Arg-p-nitroanilide. The reactive mixture (600 μl), containing 6 μg of diluted enzyme in 100 mM glycine NaOH buffer (pH 9.5) and 1 mM chromogenic substrate (100 mM in dimethylsulfoxide), was incubated at 40°C for 15 min. The reaction was followed by measuring the release of 4-nitroanilide spectrophotometrically at 405 nm and the results were expressed as relative activity, in percentage, taking the maximum value as 100%.

Effect of inhibitors and other compounds on the enzyme activity

Effect of inhibitors on the enzyme was determined by its preincubation (15 min, 40°C) in the presence of 5 mM phenylmethanesulfonyl fluoride (PMSF), 1 or 10 mM 1,10-phenanthroline and tetrasodium ethylenediaminetetraacetate (EDTA), 1 or 5 mM leupeptin, chymostatin, pepstatin, soybean trypsin inhibitor and aprotinin. After preincubation, an aliquot was removed and added to the protease assay mixture. Stock solutions of the protease inhibitors were prepared either in water (EDTA, leupeptin, aprotinin and soybean trypsin inhibitor) or in ethanol (PMSF, pepstatin and 1,10-phenanthroline) or in dimethylsulfoxide (chymostatin).

Effect of substances on protease activity was tested in presence of 0.5 and 5% sodium dodecylsulfate (SDS), ethanol, Tween 80 and dimethylsulfoxide, or 10 and 100 mM sodium carbonate, sodium acetate, sodium citrate and sodium phosphate. The enzyme was preincubated at 40°C for 15 min with each substance, followed by protease activity assay. Results were expressed as activity inhibition, in percentage, taking activity in the absence of modifiers as 100%.

N-terminal amino acid sequence

Purified enzyme was freeze-dried and submitted to amino acid sequence analysis in the Biochemistry and Molecular Biology Laboratory of the Federal
University of São Carlos (UFSCAR), São Paulo, Brazil. The enzyme was transferred to PVDF membrane and subjected to automated amino-acid sequence analysis using the Edman degradation method, in a PPSQ-23 Protein Sequencer (Shimadzu).

Results and discussion

The protease active at alkaline pH produced by A. clavatus was purified to apparent electrophoretic homogeneity by the ammonium sulfate precipitation and molecular exclusion chromatography on Sephadex G-75. The elution resulted in one peak of proteolytic activity (Fig. 1). Alkaline protease showed a specific activity of 131.41 U mg\(^{-1}\) protein, recovery of 22.1% and the purification factor was 4.04 (Table 1). SDS-PAGE analysis of the purified enzyme showed a molecular mass of about 35 kDa (Fig. 2). Molecular masses of microbial proteases are generally from 18 to 35 kDa (Gupta et al. 2002).

The enzyme showed an optimum activity temperature of 40°C (Fig. 3A) and half-lives of 100, 25 and 18 min at 40, 45 and 50°C, respectively. The protease was unstable above this temperature. Several alkaline proteases from Aspergillus species have an optimum temperature at 40°C (Pekkarinen et al. 2002). Calcium at concentration of 5 mM increased the thermal stability of the enzyme. After 60 min of incubation in the presence of Ca\(^{2+}\) at 50°C, the remaining activity was 25%, whereas in the Ca\(^{2+}\) absence it was 6%. The

![Fig. 1](image1.png)

![Fig. 2](image2.png)

**Table 1** Purification of the alkaline protease from A. clavatus

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein mg</th>
<th>Total activity U</th>
<th>Specific activity U/mg protein</th>
<th>Recovery %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>21.00</td>
<td>682.50</td>
<td>32.50</td>
<td>100.0</td>
<td>1.00</td>
</tr>
<tr>
<td>40–75% (NH(_4))(_2)SO(_4)</td>
<td>9.73</td>
<td>319.97</td>
<td>32.88</td>
<td>46.9</td>
<td>1.01</td>
</tr>
<tr>
<td>Sephadex G-75 pH 6.8</td>
<td>1.15</td>
<td>151.12</td>
<td>131.41</td>
<td>22.1</td>
<td>4.04</td>
</tr>
</tbody>
</table>

Data are the mean of three different preparations.
Slight activity (26% at 1 mM) was observed with N1 mM, 100%), and significant activity was observed with Tos-Gly-Pro-Arg-
N-nine residue attached to the carboxyl side of establish that the protease acts specifically on the argi-

Results with various chromogenic substrates clearly establish that the protease acts specifically on the argi-

half-life at 60°C, in the presence of Ca2+, was 6 min. Calcium addition also increased the stability of alkaline proteases from Aspergillus tamarii (Boer and Peralta 2000) and it is recognized as a stability factor (Zamost et al. 1990). Optimum pH was 9.5 at 40°C and the enzyme was stable over a wide range of pH from 6 to 11 (Fig. 3B). Using casein as substrate (1–45 g l–1), a \( K_m \) value of 2.9 mg ml–1 and \( V_{\text{lim}} \) value of 83.3 U mg–1 protein were observed. The double reciprocal graphic representation was linear, confirming the Michaelian behavior of this protease.

Results with various chromogenic substrates clearly establish that the protease acts specifically on the argi-

Results of sensitivity of purified protease to various protease inhibitors and other substances showed that total inhibition of casein hydrolysis was achieved by pre-incubation of the enzyme with PMSF, chymostatin and pepstatin at low concentrations. The high sensitivity of protease to inactivation by PMSF and chymostatin confirming that the enzyme belongs to a serine proteases group and it is a subtilisin-like, sup-

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Kundu S, Sundd M, Jagannadham MV (2000) Purification and characterization of a stable cysteine protease ervatamin B, with two disulfide bridges, from the latex of Ervatamia ulni (88%) and Metarhizium anisopliae (81%). This is another strong indication that this enzyme belongs to a serine proteases group and it is a subtilisin-like, sup-
porting the observations made with the specific inhib-
itors and substrates specificity. This classification could be definitively confirmed by determining its entire amino-acid sequence.


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Fig. 3 Effect of temperature (a) and pH (b) on activity (●) and stability (○) of alkaline protease from Aspergillus clavatus. a: McIlvaine (pH 2.0–8.0), 100 mM glycine-NaOH (pH 9.5–10.5) and sodium phosphate (pH 11–12) buffers, 40°C

