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Full Length Research Paper

Extracellular acid protease from *Aspergillus niger* I1: purification and characterization

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A new strain of *Aspergillus niger* producing acid protease was isolated and identified by universal primers NL1 and NL4. The acid protease from *A. niger* I1 was purified to homogeneity by ultrafiltration using a 10-KDa cut-off membrane, gel filtration on Sephadex G-75 and ion exchange chromatography on CM-Sephadex C-50, with a 3.55-fold increase in specific activity and 56% recovery. The molecular weight of the protease was estimated to be 50 kDa on SDS-PAGE and gel filtration, which is higher than those from other *A. niger* strains. Carbohydrate content of the purified protease, determined by the chemical anthrone method, was calculated to be 16%. The *K*m and Vmax for caseinolytic activity of the purified enzyme were found to be 1.02 mM and 2.2 µmol/min, respectively. The enzyme was optimally active at 60°C and pH 3.0. The most metal ions tested had no significant effect on protease activity. The enzyme activity was inhibited by pepstatin A, suggesting that the purified enzyme is an aspartic protease.

Key words: Acid protease, *Aspergillus niger*, purification, aspergillopepsin, glycosylation.

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes accounting for about 60% of the total industrial market (Rao et al., 1998). They have diverse applications in a wide variety of industries, such as in detergent, food, pharmaceutical, leather, silk and for recovery of silver from used X-ray films (Ward, 1983; Gupta et al., 2002). The industrial demand for highly active preparations of proteolytic enzymes with appropriate specificity and stability to extreme pH and temperature continues to stimulate the search for new enzyme sources.

Proteases with high activity and stability in acid pH range have important industrial applications, especially in the food processing industry, such as dairy industry as milk clotting agents for the manufacture of cheese (Sumantha et al., 2006) or to improve food flavours (Ishibashi et al., 1988). Acid proteases have been isolated and characterized from mammals, plants, bacteria and fungi (Wu and Hang, 1998). A considerable number of *Aspergilli* species are known to produce extracellular acid proteases such as *Aspergillus niger* (O'Donnell et al., 2001), *A. oryzae* (Tsujita and Endo, 1978), *A. awamori* (Moralejo et al., 2002), *A. fumigatus* (Reichard et al., 1995) and *A. saitoi* (Tello-Solis and Hernandez-Arana, 1995). These enzymes are predominantly extracellular, isolated in active form from the culture medium and some of them are available in commercial scale (Rao et al., 1998). Several fungal acid proteases called aspergillopepsin have been purified and characterized from *Aspergillus* strains (Ishishima, 2004; Percin et al., 2009). Their molecular weight have been reported to range from 30 to 40 kDa and showed maximal activity at pH 3.0 to 4.0 (Rao et al., 1998). *A. niger* is known to produce five different endopeptidases (Van den Hombergh et al., 1997), two carboxypeptidases (Dal Degan et al., 1992) and one aminopeptidase (Basten et al., 2001). The two major extracellular acid proteases are called proctases A and B. Proctase A (20 kDa), a non-pepsin type protease is not inhibited by pepstatin (Takahashi et al., 1991), whereas proctase B or

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aspergillopepsin I (35 kDa) which is inhibited by pepstatin belonging to the A1 family of proteases (Inoue et al., 1995; 1996).

The isolation and screening of microorganisms from naturally occurring acid habitats are expected to provide new strains producing enzymes active and stable in acid conditions. This paper deals with the purification and characterization of an acid protease produced by *A. niger* I1 strain newly isolated from olive oil mill wastewater.

MATERIALS AND METHODS

Chemicals

Casein, pepstatin A, ethylenediaminetetraacetic acid (EDTA), glycine, trichloroacetic acid (TCA), molecular mass markers were purchased from Sigma Chemical Co. (St. Louis MO, USA). Sodium dodecyl sulphate (SDS), acrylamide, ammonium per-sulphate, tetramethylethylenediamine and Coomassie brilliant blue R250 were from Bio-Rad Laboratories (France). Supports of chromatography used for protease purification: Sephadex G-75 and CM-Sephadex C-50 were from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

Microorganism and primers

The strain used throughout this study was isolated from olive oil mill wastewater. The isolate was identified as *A. niger* with universal primers NL1 (5'GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'GGTCCGTGTTTCAAGACGG). The strain was maintained on potato-dextrose-agar plates at 30°C. Spores for inoculum were prepared from 7 days-old colonies by flooding with 10 ml of sterile distilled water and scraping off the agar plates.

Culture and growth conditions

The medium used for protease production by *A. niger* I1 strain was composed of (g/l): $CaCl_2$ 7.H_2O 0.4, KH_2PO_4 7.0, Na_2HPO_4 2.5, $MgSO_4$ 7. H_2O 0.5, $ZnCl_2$ 0.1, NaCl 0.3, fish (*Sardinella aurita*) flour 5, Hull grain of wheat 10; pH 6.0. Media were autoclaved at 120°C for 20 min. Cultures were inoculated with 10^7 spores/ml and incubated in a rotatory shaker at 150 rpm for 72 h at 30°C, in 300 ml Erlenmeyer flasks with a working volume of 50 ml. The cultures were centrifuged at 10,000 rpm for 10 min at 4°C to remove fungi mycelia and supernatants were used as the enzyme solution. All experiments were carried out in duplicate and repeated at least twice.

Assay of protease activity

Protease activity was measured by the method of Kembhavi et al. (1993), using casein as a substrate. Half milliliter of the enzyme, suitably diluted, was mixed with 0.5 ml of 100 mM glycine-HCl (pH 3.0) containing 1% casein, and incubated for 5 min at 60°C. The reaction was stopped by addition of 0.5 ml trichloroacetic acid (20%. w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 rpm for 15 min and the precipitate was removed. The absorbance was measured at 280 nm using a UV spectrophotometer. A standard curve was generated using solutions of 0-50 mg/l tyrosine. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine in one minute under the experimental conditions used. A control lacking the enzyme was included in each assay.

Protein content

The protein content of individual fractions, obtained after different steps of chromatography, was monitored by measuring the absorbance at 280 nm. Quantitative estimation of protein was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Purification of acid protease

Ultrafiltration: The crude supernatant (190 ml) was concentrated by ultrafiltration with a 10-kDa molecular weight cut-off membrane (MSI Miscron Separations inc., Webstboro, MA, USA) in an Amicon Model 8050 stirred ultrafiltration cell (Amicon corp., Danvers, USA). The pressure of the cell was kept at 75 psi by nitrogen gas.

Sephadex G-75 filtration

The concentrated extract was then subjected to gel filtration on a Sephadex G-75 column (3 cm x 90 cm) previously equilibrated with 25 mM glycine-HCl buffer pH 3.0. Fractions of 4.0 ml were collected at a flow rate of 25 ml/h after elution with the same buffer. Protein content (abs. at 280 nm) and protease activity in each fraction were measured. Fractions showing protease activities were pooled.

CM-Sephadex C-50 separation

The active fractions were applied to a CM-Sephadex C-50 column (3 cm x 30 cm) equilibrated with 25 mM glycine-HCl buffer, pH 3.0. After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0-0.5 M in the equilibrating buffer. Fractions (6.0 ml each) were collected at a flow rate of 96 ml/h, and analyzed for protease activity and protein concentration. Active fractions were pooled and stored at 4°C for further analysis. All the purification steps were carried out at temperature not exceeding 4°C.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was carried out to determine the purity and molecular weight of the enzyme, as described by Laemmli (1970). The molecular weight of the enzyme was estimated using a low-molecular weight calibration kit as markers consisting of bovine serum albumin (66 kDa), egg white ovalbumin (45 kDa), glyceraldehyde-3-P deshydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine α-lactalbumin (14.2 kDa). Protein bands were visualized after staining with Coomassie brilliant blue R-250.

Detection of proteolytic activity on polyacrylamide gels

Zymography was performed in conjunction with SDS-PAGE according to the method described by Garcia-Carreno et al. (1993) with slight modification. The sample was not heated before loading in the gel. After electrophoresis, the gel was submerged in 100 mM glycine–HCl buffer (pH 3.0) containing 2.5% Triton X-100 for 30 min at 4°C, with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with 100 mM glycine–HCl buffer (pH 3.0). The gel was then incubated with 1% (w/v) casein in 100 mM glycine–HCl buffer (pH 3.0) for 30 min at 50°C. Finally, the gel was stained with Coomassie Brilliant Blue

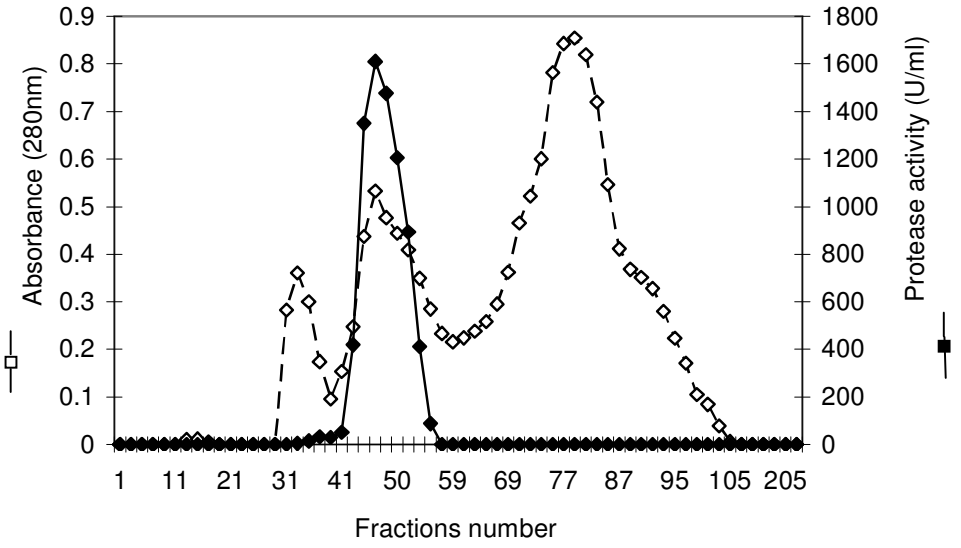

Figure 1. Purification profile of acid protease from *Aspergillus niger* I1 by gel filtration on Sephadex G-75 column. The concentrated enzyme preparation was applied to a 3 x 90 cm column, equilibrated and eluted with 25 mM glycine-HCl (pH 3.0) buffer at a flow rate of 25 ml/h.

R-250 for zymography analysis. The development of clear zones on the blue background indicated the presence of protease activity.

Effects of pH and temperature on the activity and stability of the enzyme

The effect of pH on protease activity was performed at 60°C in different buffers at 100 mM. For the determination of pH stability, the enzyme was kept at 4°C for 1 h in buffers of different pH and then the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: glycine-HCl buffer for pH 2.0-2.5-3.0-3.5, acetate buffer, for pH 4.0, and phosphate buffer for pH 6.0-8.0.

To study the effect of temperature, the activity was tested at different temperatures using casein as a substrate for 5 min at pH 3.0. Thermal stability was examined by incubating the purified enzyme 60 min at 30, 40, 50 and 60°C. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 3.0 and 60°C. The non-heated enzyme was considered as a control (100%).

Effects of metal ions and enzyme inhibitors on protease activity

The effect of various metal ions at 5 mM on enzyme activity was investigated using $CaCl_2$, $MnSO_4$, $ZnSO_4$, $CoSO_4$, $CuSO_4$, $BaCl_2$, $FeCl_2$, $HgCl_2$, $MgSO_4$, NaCl and KCl.

The effects of enzyme inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), pepstatin A, β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA). The purified enzyme was preincubated with inhibitors for 60 min at 4°C and then the remaining enzyme activity was estimated using casein as a substrate. The activity of the enzyme without any additives was taken as the control.

Kinetic studies

The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of the acid protease for casein were determined at different substrate concentrations. They are evaluated by plotting the data on a Lineweaver-Burk double-reciprocal graph ($1/v_i$) versus ($1/[S]$) ((Lineweaver and Burk, 1934)).

Quantification of carbohydrate

Carbohydrate was quantified with the anthrone method (Dregwood, 1946). Protein solution (2 ml) was mixed with 4 ml of 0.2% anthrone in concentrate sulfuric acid. The samples were kept at 100°C for 4 min (instead of 16 min (Dregwood, 1946)). This reduction of incubation time helped to overcome problems with coloured products that would result after prolonged exposure of the sample to sulfuric acid at 100°C. The glucose standard solution was treated identically. The optical density at 625 nm of the samples was read within 1 h.

RESULTS

Purification of *A. niger* I1 protease

Acid protease from *A. niger* I1 was purified by three-step procedure described in section 2. In the first step, the culture supernatant was concentrated by ultrafiltration using a 10-kDa membrane. This step resulted in a 1.25-fold purification with 70% recovery. The concentrated enzyme was then subjected to gel filtration on a Sephadex G-75 column. The elution profiles of proteins and protease activity are shown in Figure 1. This procedure yielded one peak of protease activity. Fractions containing protease activity (fractions 43 to 59) were pooled and then loaded on a CM-Sephadex C-50 column (Figure 2). Bound proteins were eluted with a linear gradient of NaCl (0-0.5 mM). Fraction 115, which showed the highest activity, was analysed on a 12% SDS-

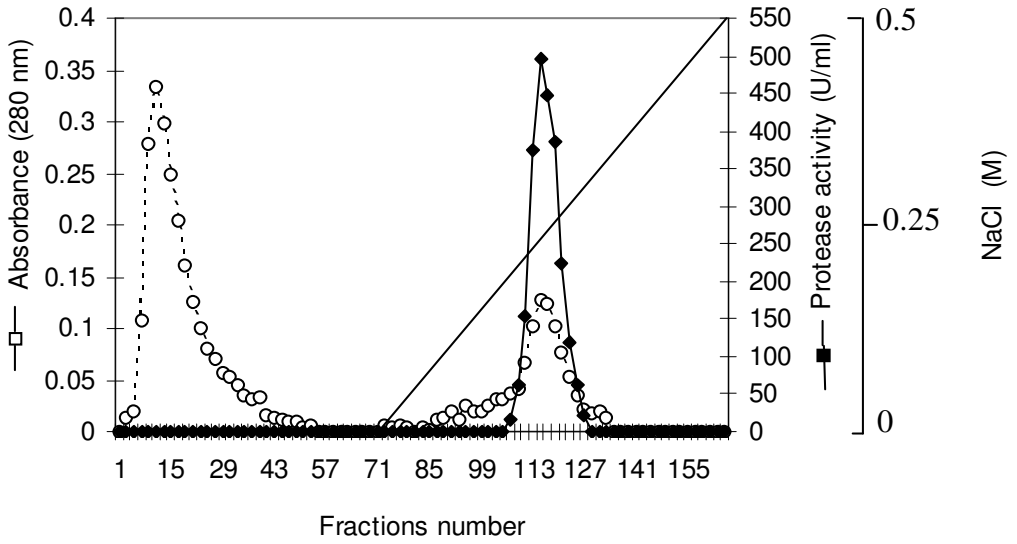

Figure 2. Elution profile of *A. niger* I1 protease from a CM-sephadex C-50 column. The enzyme was eluted with a linear gradient of NaCl (0-0.5 M) in glycine-HCl buffer (pH 3.0; 25 mM) at a flow rate of 120 ml/h.

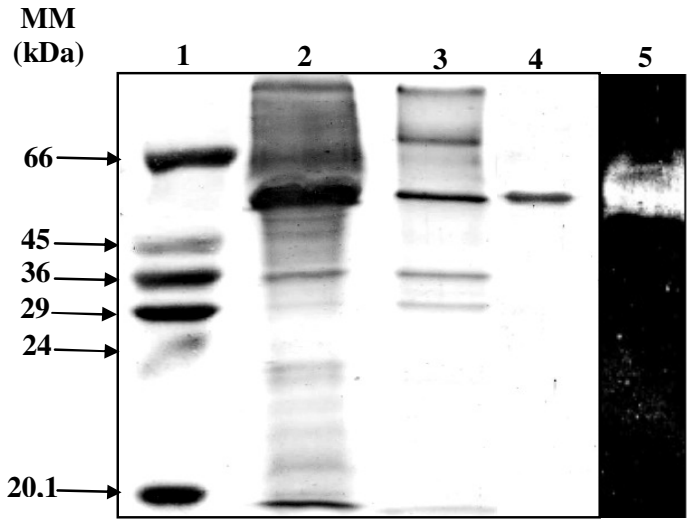

Figure 3. SDS-PAGE of the purified protease from *A. niger* I1. Lane 1: Molecular weight markers; lane 2: crude enzyme extract; lane 3: pool filtered by G-75 column; lane 4: purified acid protease after elution by CM-sephadex; lane 5: zymography of purified protease.

polyacrylamide gel electrophoresis. As shown in Figure 3, a unique protein band was obtained. The molecular weight of the *A. niger* I1 protease was estimated to be 50 kDa by SDS-PAGE using molecular weight markers.

Purity of the enzyme was also evaluated using zymogram activity staining. As shown in Figure 3, a unique clear band of proteolytic activity was observed in the gel indicating the homogeneity of the purified protease. Taken together, all these results indicate that the protease is a monomeric protein. The purified enzyme was characterized for its K_m and V_{max} towards casein as a substrate. It showed a K_m of 1.02 mM and a V_{max} of 2.2 µmol/min.

The results of the purification procedure are summarized in Table 1. After the final purification step, the protease was purified 3.55-fold with a recovery of 56.29% and a specific activity of 13429 U/mg of protein.

Effect of pH on enzyme activity and stability

The effect of pH on protease activity of the purified enzyme using casein as a substrate was studied at various pH values at 60°C. The pH profile of the purified protease is shown in Figure 4a. The enzyme was most active at pH 3.0 and protease activity decreased significantly below and above pH 3.0 and was about 40 and 55% at pH 2.5 and 3.5, respectively.

The pH stability profile of *A. niger* I1 protease was determined by the measurement of the residual activity at pH 3.0 after incubation for 60 min at various pH. As shown in Figure 4b, the purified enzyme was highly stable at pH 3.0. However, the protease retained only 11 and 40% of its activity at pH 2.0 and 4.0, respectively, and lost all of its activity at pH 7.0.

Effect of temperature on the activity and stability of the enzyme

The effect of temperature on the activity of *A. niger* I1 protease was examined at various values for 5 min at pH 3.0. As shown in Figure 5a, the *A. niger* I1 enzyme was

Table 1. Summary of the purification of *A. niger* I1 protease.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification fold
Crude extract	94,240	24.96	3,775.6	100	1
Ultrafiltration	66,100	13.95	4,739	70.14	1.25
Sephadex G-75	61,689.5	8.34	7,396.8	65.45	1.96
CM-Sephadex C-50	53,047.7	3.95	13,429.8	56.29	3.55

All operations were carried out at 4°C.

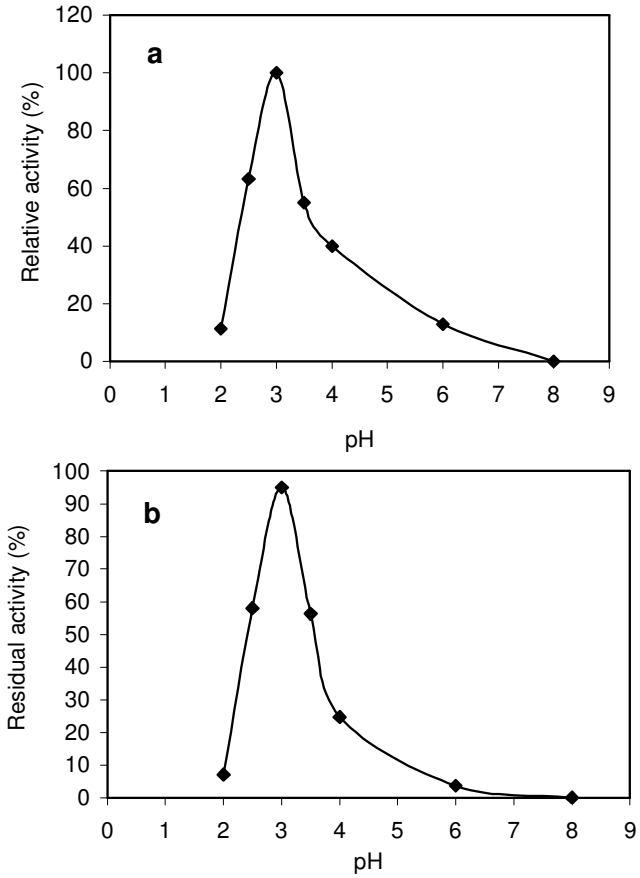

Figure 4. Effect of pH on activity (a) and stability (b) of the purified protease from *A. niger* I1. The protease activity was assayed in the pH range of 2.0-8.0 using buffers of different pH values at 60°C.The maximum activity obtained at pH3.0 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 1 h at 4°C and the residual activity was measured at pH 3.0 and 60°C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2.

active between 30 and 70°C with an optimum around 60°C. The relative activities at 50 and 70°C were about 82 and 70%, respectively.

To examine the thermal stability of *A. niger* I1 protease, the purified enzyme was incubated at pH 3.0 and at various temperatures for different time periods, and then the residual activity was measured. The thermal stability profile of the purified protease showed that the enzyme is highly stable at temperatures below 40°C (Figure 5b). At 30°C, the enzyme remains fully active even after 1 h incubation. The enzyme was rapidly inactivated at 50 and 60°C, loosing 73 and 91% of the initial activity after 15 min incubation, respectively.

Effects of metal ions

The effects of various metal ions at a concentration of 5 mM on the activity of *A. niger* I1 protease was studied at pH 3.0 and 60°C by the addition of the respective cations to the reaction mixture (Table 2). Most of the metal ions are without effect or slightly decreased the enzyme activity, while Cu^{2+} inhibited the activity by 32%.

Effect of enzyme inhibitors

The effect of a variety of enzyme inhibitors, such as chelating agent and specific group reagents on the activity was also investigated (Table 3). The purified enzyme was completely inhibited by pepstatin A, indicating the presence of aspartate at its active site. PMSF (serine protease inhibitor), EDTA (metalloenzyme inhibitor) are practically without influence on the activity of the purified enzyme.

Carbohydrate content

Carbohydrate content of the purified enzyme, determined by the chemical Anthrone method, is calculated to be 16%.

DISCUSSION

Several fungi strains producing extracellular proteases were screened from different biotopes. Of these, isolate I1 exhibiting a large zone of hydrolysis on skim milk agar plates was selected. In order to identify I1 strain, the internal transcribed spacer (ITS) region of 18S rDNA was amplified. The ITS nucleotide sequence was analyzed with the GenBank database using BLAST program and showed that I1 is an *A. niger* strain.

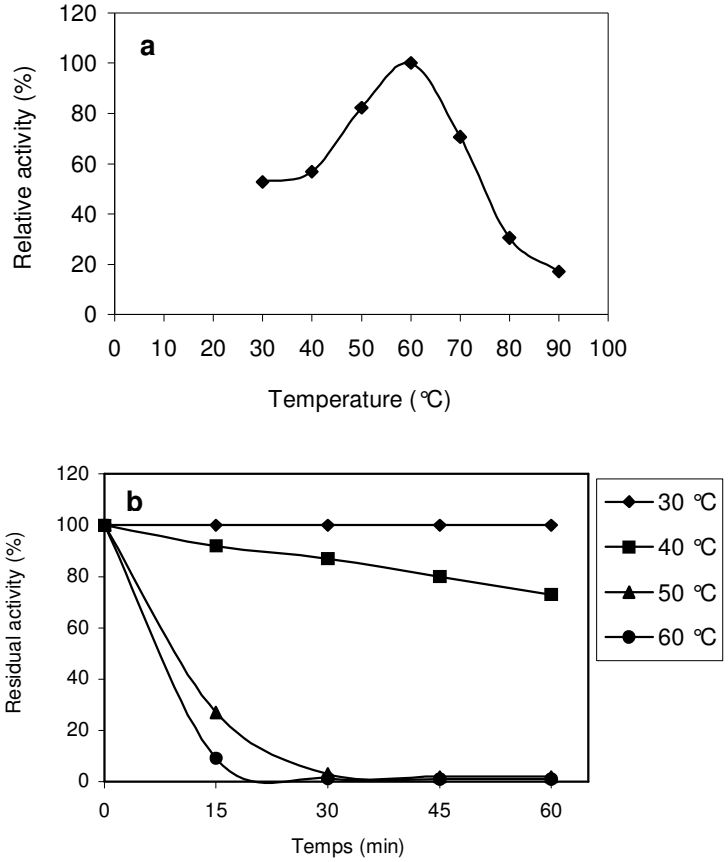

Figure 5. Effect of temperature on activity (**a**) and stability (**b**) of the purified protease from *A. niger* I1. The temperature profile was determined by assaying protease activity at temperatures between 30 and 90°C. The activity of the enzyme at 60°C was taken as 100%. The temperature stability was determined by incubating the purified enzyme at different temperature from 30 to 60°C for 60 min. The residual enzyme activity was measured under the standard assay conditions. The non-heated enzyme was considered as control (100%).

Table 2. Effect of various metal ions on acid protease activity.

Metal ions (5 mM)	Relative activity (%)
None	100
Ca^{2+}	92
Mg^{2+}	89
Zn^{2+}	92
Cu^{2+}	68
Mn^{2+}	90
Ba^{2+}	99
Co^{2+}	94
Hg^{2}	99
Fe^{2+}	99
Na^{+}	91
K^{+}	99

The activity of A1 I1 protease was determined by incubating the enzyme in the presence of various metal ions at pH 3.0 and 60°C.

Table 3. Effect of enzyme inhibitors on acid protease activity.

Inhibitors	Concentrations (mM)	Residual activity (%)
None	-	100
PMSF	5	100
EDTA	5	100
β-mercaptoethanol	5	100
Pepstatin A	$1.5 .10^{-3}$	0

The remaining protease activity was measured after pre-incubation of the purified enzyme with each inhibitor for 30 min at room temperature. Enzyme activity measured in the absence of any inhibitor was taken as 100%.

The main proteolytic activity of *A. niger* I1 was purified from the culture supernatant to homogeneity using ultra-filtration, Sephadex G-75 gel filtration and CM-Sephadex C-50 ion exchange chromatography, with a 3.55-fold increase in specific activity and 56% recovery. The purified acid protease from *A. niger* I1 is a monomeric protein with a molecular weight of 50 kDa as estimated by both Sephadex G-75 gel filtration and SDS-PAGE. The molecular weights of acid proteases from other fungi have been reported to range from 35 to 56 KDa (Morimura et al., 1994), however, no acid protease from *A. niger* strains having a molecular weight higher than 40 KDa, was described.

The enzyme was most active at pH 3.0 and protease activity decreased significantly below and above pH 3.0. Optimum pH values between 3.0 and 5.5 have been reported for protease activities of other fungi, such as *Penicillium camembertii*, pH 3.5 (Chrzanowska et al., 1995) and *Rhizopus oryzae*, pH 5.5 (Kumar et al., 2005). The optimum pH of *A. niger* I1 protease was lower than that of *A. niger* NRRL 1785 protease which exhibited an optimum at pH 4.0 (Olajuyigbe et al., 2003).

The purified enzyme showed maximum activity at 60°C. The optimum temperature of *A. niger* I1 was similar to those from other fungi proteases, such as *R. oryzae* (Kumar et al., 2005), *P. duponti* K1014 (Hashimoto et al., 1973), *P. oxalicum* (Hashem 1999) and *Cryptococcus albidus* (Alessandro and Federico, 1980) and higher than proteases from *Neosartorya fischeri* var. *spinosa* IBT 4872 (Wu and Hang, 1998) and *A. niger* NRRL 1785 which exhibited optimum temperature at 50°C (Olajuyigbe et al., 2003).

The effects of various metal ions at a concentration of 5 mM on the activity of *A. niger* I1 protease was studied at pH 3.0 and 60°C. *A. niger* I1 acid protease was practically insensitive to the most metallic ions. So this property is in the view of potential food industry applications. None of the metallic ions enhanced protease activity. In this respect, the *A. niger* I1 enzyme resembles to the acid proteases of *Hebeloma crustuliniforme* (Zhu et al., 1990) and *Mucor pusillus* (Somkuti and Babel, 1968). The effect of a variety of enzyme inhibitors, such as chelating agent and specific group reagents on the activity was also investigated. Among the eight types of cited proteases produced by *A. niger* strains (Van den Hombergh et al., 1997; Basten et al., 2001; Monod et al., 2002), the purified enzyme can be classified as an acid (aspartic) protease and belonged to the A1 family because of its optimal pH at 3.0 and full inhibition by pepstatin A (Gomi et al., 1993).

Conclusion

This paper describes the purification and characterization of *A. niger* I1 strain acid protease. The enzyme was purified from the culture supernatant by three steps with a 3.55-fold increase in specific activity and 56.29% recovery. The molecular weight of the purified *A. niger* I1 acid protease was determined to be 50 kDa by SDS-PAGE. The optimal pH and temperature for the enzyme activity were pH 3.0 and 60 °C, respectively. The protease of *A. niger* I1 can be classified as an aspartic protease belonging to the family A1 because of its optimal pH and inhibition by pepstatin A.

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