



## Characterisation of acid protease expressed from *Aspergillus oryzae* MTCC 5341

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### ABSTRACT

*Aspergillus oryzae* (MTCC 5341) has the largest expanse of hydrolytic genes, that includes 135 protease genes coding for alkaline, acid as well as neutral proteases. This study reports the purification and characterisation of an acid protease obtained from *A. oryzae* MTCC 5341. *A. oryzae* MTCC 5341 produces one of the highest reported acid protease activities reported so far ( $8.3 \times 10^5$  U/g dry bran). The extracellular acid protease (47 kDa) was found to be active in the pH range 3.0–4.0 and stable in the pH range 2.5–6.5. Optimum temperature for activity was 55 °C. The protease was purified 17-fold with a yield of 29%. The enzyme was characterised to be an aspartate protease by inhibition studies, using pepstatin and its ability to activate trypsinogen. The enzyme cleaved the B-chain of insulin at L–V and Y–T residues.

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### 1. Introduction

Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism and active site (Sumantha, Larroche, & Pandey, 2006). Acid proteases (E.C. 3.4.23.) are endopeptidases, with molecular masses in the range 30–45 kDa, that depend on aspartic acid residues for their catalytic activity and show maximal activity at low pH. Microbial aspartic proteases can be broadly divided into pepsin-like enzymes and rennin-like enzymes (Rao, Tanksale, Ghatge, & Deshpande, 1998). Acid proteases offer a variety of applications in the food and beverage industry and medicine. The worldwide requirement for these enzymes with specific applications is increasing rapidly. Although several reports of acid protease from fungal sources have appeared in the recent past, the enzymes have low reaction rates and are not well characterised. With the advent of modern biotechnological tools, it is possible to produce these enzymes in bulk quantities for use as crude preparations in food industries.

*Aspergillus oryzae* has been selected as a non-toxicogenic strain, due either to its long history of industrial use or to its evolution. *A. oryzae* is genomically well characterised and considered to be a “safe” organism for production of food enzymes as it lacks expressed sequence tags for the genes responsible for aflatoxin production. Accordingly *A. oryzae* has eight chromosomes of 37 MB genome size, comprising 12,074 genes, which encode proteins with length is greater than 100 amino acids. Molecular history of the organism shows that *A. oryzae* has the largest expansion of hydro-

lytic genes (135 proteinase genes). In contrast to the overall increase in the number of proteinases, *A. oryzae* has fewer glycosyl hydrolases with, either a cellulose-binding domain or a starch-binding domain to digest insoluble cellulose or raw or granular starch, making *A. oryzae* a major producer of proteases (Machida et al., 2005).

*A. oryzae* possesses more secretory proteinase genes that function in acidic pH, reflecting *A. oryzae*'s adaptation to acidic pH during the course of its domestication. *A. oryzae* grows on the surface of solid materials such as steamed rice, ground soybean or agricultural byproducts, e.g. wheat bran, rice bran, bagasse and many other substrates, where amino acids and sugars are initially deficient. This has driven the organism to have a number of hydrolytic enzymes and transport-related gene families to efficiently utilise external nutrient sources for their growth (Ferea, Bostien, Brown, & Rosenzweig, 1999).

The present study focuses on the isolation of a high activity acid protease and its characterisation. The enzyme has one of the highest reported specific activities.

### 2. Materials and methods

#### 2.1. Materials

Haemoglobin (acid denatured) was procured from MP Biomedical Inc. (Solon, Ohio). Trichloroacetic acid was from Himedia Laboratories Pvt. Ltd (Mumbai, India). Acrylamide, bis-acrylamide, sodium dodecyl sulfate, TEMED,  $\beta$ -mercaptoethanol, insulin chains (A and B), trypsinogen and trypsin were procured from Sigma Chemical Co., USA. Molecular weight marker kit S7

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(14.2–97.4 kDa) was procured from Bangalore Genie, India. *A. oryzae*, isolated from the koji of soy sauce, is deposited at the Microbial Type Culture Collection, Chandigarh, India under the accession number MTCC 5341. All other chemicals used were of analytical grade.

## 2.2. Production of acid protease by solid-state fermentation

Acid protease was produced extracellularly when *A. oryzae* was grown on wheat bran as medium with 60% added moisture. Acidic pH condition was maintained by the addition of a mineral solution containing trace salts  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (70 mg each in 100 ml of 0.2 N HCl). Defatted soy flour (4%) was added to the bran as a source of additional protein. Spore suspension ( $10^5$  spores/ml, 1 ml/100 g bran) was added as inoculum and the bran incubated at 27 °C and 80% R.H. The mouldy bran was harvested at the end of 120 h of fermentation, dried and stored at 4 °C. Extracellular proteases were extracted from the mouldy bran by water containing 0.1 M sodium chloride (1:10, bran:solvent) for 1 h without stirring at 4 °C.

## 2.3. Assay method

Acid protease activity was determined by following the procedure described by Tello-Solis, Rodriguez-Romero, and Hernandez Arana (1994). Proteolytic activity was determined, using 2% haemoglobin (acid denatured) in glycine-HCl (pH 3.2, 0.1 M) as substrate. One millilitre of substrate was incubated with 400  $\mu\text{l}$  of appropriately diluted enzyme solution for 10 min at 55 °C. After 10 min, 2 ml of 5% TCA were added and the tubes were incubated for 20 min to allow unhydrolysed protein to settle. Unhydrolysed protein was removed by filtration (Whatman No. 1). Absorbance of the supernatant was measured at 280 nm. One unit is defined as the amount of enzyme that produces an increase in absorbance of 0.001 per min under the above conditions. Protein concentration was determined (Lowry, Rosenbrough, Farr, & Randall, 1951) using bovine serum albumin as standard.

## 2.4. Purification of acid protease

The crude enzyme extract, obtained after extracting mouldy bran with water containing 0.1 M NaCl, was precipitated with chilled acetone (1:2, extract:acetone). Two volumes of chilled acetone were slowly added to the extract and the precipitate was allowed to settle for 4 h. The precipitated protein was separated by centrifuging at 6000 rpm for 20 min. The pellet was dissolved in minimal volumes of 50 mM phosphate buffer, pH 6.0 and dialysed against the same buffer to remove trace amounts of acetone. The dialysate was applied onto a DEAE-cellulose ion-exchange column (2.5  $\times$  50 cm, 50 ml). Bound protein was eluted with a salt gradient of 0–0.5 M NaCl. The active fractions were concentrated by ultrafiltration (nominal weight cut-off membrane 10,000 Da, PLGC low protein binding membrane, Millipore Inc., USA), followed by molecular sieve chromatography with G-75 superfine (1  $\times$  100 cm, 100 ml), using 50 mM phosphate buffer (pH 6.0). The active fractions were pooled and the homogeneity was confirmed by SDS-PAGE and HPLC.

## 2.5. Homogeneity of acid protease

### 2.5.1. Gel electrophoresis

Native PAGE was performed, using 12.5% (w/v) acrylamide in gels in the absence of sodium dodecyl sulphate and  $\beta$ -mercaptoethanol.

For activity staining, electrophoresis was performed on 12.5% gels in the native condition (without SDS and  $\beta$ -mercaptoethanol)

containing 1% copolymerised gelatin in the separating gel, as described previously (Heussen & Dowdle, 1980). After the run, the gel was incubated in glycine-HCl buffer (3.2 pH) at 50 °C for 30 min. Proteolytic activity *in situ* was visualised as transparent bands against a blue background when stained with 0.1% amido black.

SDS-PAGE was performed using 12.5% (w/v) acrylamide in gels, as described previously (Laemmli, 1970). Protein bands were visualised by silver staining. The molecular weight marker standards used were phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa).

### 2.5.2. Size exclusion chromatography on HPLC

HPLC was carried out using a TSK G 2000 SW gel column (7.8 mm  $\times$  300 mm, 5  $\mu\text{m}$ ) at a flow rate of 0.2 ml/min. The column was pre equilibrated with 50 mM phosphate buffer, pH 6, with 0.3 M NaCl. Elution was carried out with the same buffer. Purified enzyme was injected and detection was monitored at 280 nm. Molecular weight markers used were alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

## 2.6. Characterisation of protease from *A. oryzae*

### 2.6.1. pH optimum and stability and temperature optimum for activity

The pH optimum for activity of protease was determined by incubation in buffer in the pH range 2–10. Buffers used were 0.1 M glycine-HCl (2–5 pH), 0.1 M acetate (5–7 pH) and 0.1 M glycine-NaOH (7–10 pH). The activity was assayed at 55 °C. Maximum activity obtained within the range was expressed as 100% and residual activities calculated.

pH stability of protease was carried out by incubating the enzyme for 24 h in buffer in the pH range 2–10 (same buffers as above) at 27 °C, followed by activity assay at 55 °C. Maximum activity obtained within the range was expressed as 100% and residual activities calculated.

The optimum temperature for activity was determined by assaying protease activity in the range 35–70 °C. Heat-denatured haemoglobin (2%) in 0.1 M glycine-HCl, pH 3.2, was used as substrate. The maximum activity obtained within the range was taken as 100% and the residual activities calculated.

### 2.6.2. Determination of $K_m$ and $V_{max}$

The  $K_m$  and  $V_{max}$  values for the purified acid protease were determined with acid-denatured haemoglobin as the substrate in glycine-HCl buffer, 0.1 M, pH 3.2. The enzyme was incubated with a range of 0–2% concentration of the substrate for a period of 10 min at 55 °C. A double reciprocal plot was used for estimating  $K_m$  and  $V_{max}$ .

## 2.7. Spectral characterisation of acid protease

### 2.7.1. UV absorption

The absorption spectrum of acid protease was measured using a Shimadzu UV1601 spectrophotometer. The tryptophan residues in the protein were estimated by an earlier reported NBS method (Spande & Witkop, 1967).

### 2.7.2. Fluorescence measurements

Fluorescence measurements were made using a Shimadzu RF-5000 automatic recording spectrofluorimeter, in conjunction with a constant temperature circulating water bath. The temperature of the cell was maintained at 27 °C. Protein solution having absorbance of 0.075 at 280 nm in 50 mM phosphate buffer, pH 6.0, was used. The excitation spectrum was recorded in the range 200–

300 nm, fixing the emission wavelength at 335 nm. The excitation wavelength was fixed at 278 nm (excitation maximum for the protein) and emission spectrum recorded in the region 300–400 nm. The spectra were recorded with 5 nm bandwidth for both excitation and emission monochromators.

### 2.7.3. Circular dichroism (CD) studies

CD measurements were made using a Jasco J-810 automatic spectropolarimeter fitted with a xenon lamp. The instrument was calibrated with d (+)-10-camphor-sulphonic acid ammonium salt. The lamp was purged continuously with nitrogen before and during the experiments. The scans were recorded thrice. The far UV CD spectrum was recorded between 190 and 260 nm using a 1 mm path length cell and a protein concentration of 0.22 mg/ml in 50 mM phosphate buffer, pH 4.5. The near UV CD spectrum was recorded in the range 260–320 nm using a 1 cm path length cell and a protein concentration of 1 mg/ml in 50 mM phosphate buffer, pH 4.5. Secondary structure analysis was carried out using the CDSSTR method (Compton & Johnson, 1986; Sreerama & Woody, 2000) with reference database SP175 (Lees, Miles, Wien, & Wallace, 2006) available in DICHROWEB (Lobley, Whitmore, & Wallace, 2002).

### 2.8. Thermal stability

The acid protease was incubated in 0.1 M acetate buffer, pH 5, in the temperature range 30–65 °C for 15 min. The samples were immediately cooled in ice and residual activity was assayed. The unincubated enzyme activity was taken as 100%.

### 2.9. Inhibition studies

The ability of class specific inhibitors to inhibit protease activity was studied in the range 0–100 μM. The inhibitors used include EDTA (metalloproteases), iodoacetamide (cystine proteases), PMSF and antipain hydrochloride (serine proteases) and pepstatin (aspartate protease). After incubating enzyme with the inhibitor for 30 min at 27 °C, residual activity was assayed under optimal conditions for activity. The control enzyme activity, without the inhibitor, was taken as 100%.

### 2.10. Trypsinogen activation

Trypsinogen activation was studied as reported earlier (Sodek & Hoffmann, 1982). Commercial trypsinogen was prepared at 1 mg/ml concentration in 0.1 M glycine-HCl buffer, pH 3.2. To this, different aliquots of acid protease were added and incubated at 55 °C for different time intervals. After end of each incubation time, activation was examined by trypsin activity on casein. Twenty microlitre of this were also analysed for trypsin formation through size exclusion chromatography on HPLC.

### 2.11. Cleavage specificity

Cleavage specificity of acid protease was determined by analysis of peptides in the hydrolysed insulin chains (A and B). The peptide chains of oxidised insulin (1 mg/ml) in 0.1 M glycine-HCl, pH 3.2, were subjected to hydrolysis by acid protease at 55 °C for 5 min. At the end of the incubation, the reaction was stopped by 50 μl of 0.01% TFA. Peptide fractions were separated by a Waters®HPLC system fitted with a C<sub>18</sub> column (250 × 4.6 mm, 5 μM). A nine step gradient system, as described by Stentz, Kitabachi, Schilling, Schronk, and Seyer (1989) having 0.1% trifluoroacetic acid/H<sub>2</sub>O, and acetonitrile was employed. The gradient system used was step 1: 10% ACN, 90% 0.1% trifluoroacetic acid/H<sub>2</sub>O isocratic for 10 min; step 2: 10 min gradient to 20% ACN, 80% 0.1%

trifluoroacetic acid/H<sub>2</sub>O; step 3: 10 min isocratic at 20% ACN, 80% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 4: 20 min gradient to 25% ACN, 75% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 5: 10 min isocratic at 25% ACN, 75% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 6: 10 min gradient to 28% ACN, 72% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 7: 20 min isocratic at 28% ACN, 72% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 8: 30 min gradient to 50% ACN, 50% 0.1% trifluoroacetic acid/H<sub>2</sub>O; step 9: 5 min gradient to initial conditions of 10% ACN, 90% 0.1% trifluoroacetic acid/H<sub>2</sub>O. Individual peptide peaks were collected for analysis of their molecular weights using mass spectrometry.

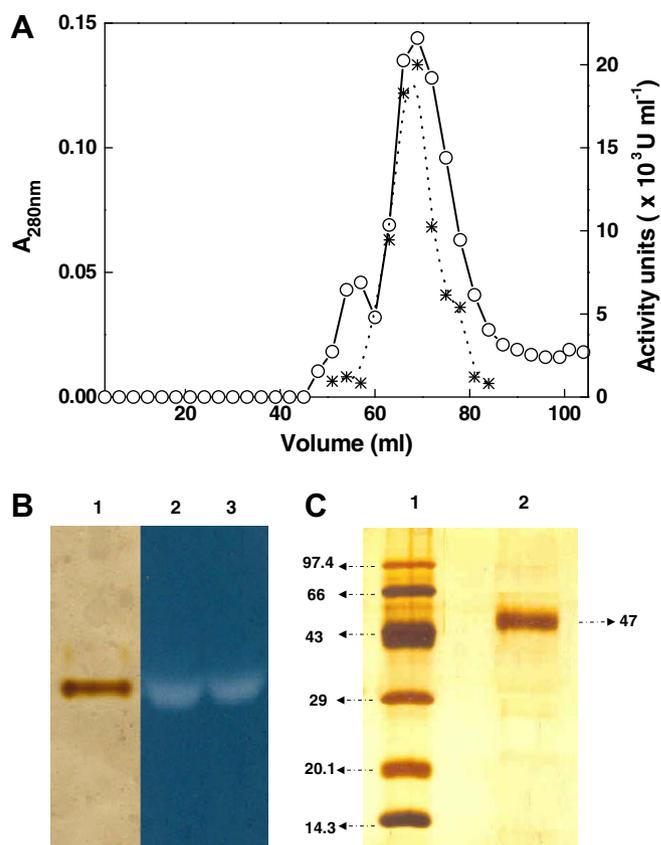
### 2.12. Mass spectrometry

The peptides collected after HPLC, were passed into the electro spray ionisation source in the negative ion mode of a Waters®HPLC mass spectrometer (Model Q-ToF ultima) for their molecular mass analysis. The cleavage points were deduced by comparing the mass of each peptide with its sequence.

## 3. Results and discussion

### 3.1. Purification of protease

The first step in purification of acid protease was acetone precipitation of the enzyme extract. Two volumes of chilled acetone were used to achieve a purification of 5-fold. The precipitate was



**Fig. 1.** Purification of acid protease from *Aspergillus oryzae* MTCC 5341. A. Purification of acid protease from *Aspergillus oryzae* by molecular sieve chromatography. ○—○, protein content; \*—\*, protease activity. Experimental details are given under Section 2. B. Native PAGE pattern and activity staining in gel electrophoresis for acid protease. Lane1 – Native gel pattern, lanes 2 and 3 – Zymogram of acid protease. C. SDS-PAGE pattern and molecular weight determination of acid protease. Lane1 – molecular weight markers and lane 2 – purified acid protease.

loaded onto a DEAE-cellulose column, using a linear gradient of 0–0.5 M NaCl. Acid protease activity eluted out at 0.3–0.35 M sodium chloride. The enzyme was purified 12 times with a yield of 66%. The active pool was fractionated on G-75 superfine column (Fig. 1A) with a yield of 29%. The acid protease was purified 17-fold with a specific activity of 43,658 U/mg (Table 1). Native PAGE ascertained homogeneity of the preparation and *in situ* activity of the acid protease in the gel is shown in Fig. 1B.

### 3.2. Homogeneity of acid protease

#### 3.2.1. Size exclusion chromatography

Acid protease eluted as a single peak after molecular sieve chromatography on a TSK G 2000 SW column by HPLC. The molecular weight of the acid protease was determined to be 47,578 kDa (results not shown), using standard proteins in the range 12.4–150 kDa.

#### 3.2.2. Gel electrophoresis

Purified acid protease moved as single band on SDS-PAGE. The protein was homogeneous and the molecular weight of acid protease was deduced to be 47,000 Da on SDS-PAGE (Fig. 1C).

### 3.3. Characterisation of acid protease from *A. oryzae*

#### 3.3.1. Effect of pH and temperature on the activity and stability of acid protease

Acid protease from *A. oryzae* MTCC 5341 was active in the pH range 3–4, with an optimum pH of 3.2 (Fig. 2A). The enzyme was stable in the pH range 2.5–6.0 at 30 °C for 24 h. The enzyme was stable in the temperature range 40–57 °C with optimum temperature for activity of 55 °C (Fig. 2B). Acid proteases from *A. oryzae* have been reported to have pH optima in the range 2.5–4.0 (Rao et al., 1998).

#### 3.3.2. Determination of $K_m$ and $V_{max}$ for acid protease

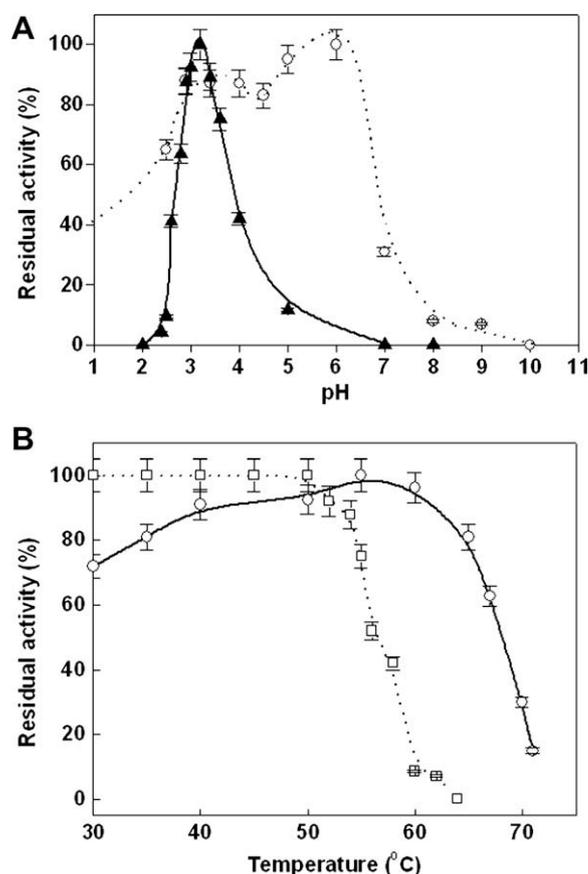
$K_m$  and  $V_{max}$  were determined to be 0.8% and 89.4  $\mu\text{mol}/\text{min}/\text{mg}$  protein, respectively, using 0–2% acid-denatured haemoglobin as substrate.

### 3.4. Spectral characterisation

The UV absorption spectrum of acid protease is shown in Fig. 3A. The enzyme has an absorption maximum at 278 nm. The number of tryptophan residues was determined to be six. The fluorescence spectrum of the enzyme exhibited an excitation maximum at 278 nm. The emission maximum was determined to be 335 nm (when excited at 278 nm), indicating that the tryptophan residues are in a fairly hydrophobic environment (Fig. 3B).

The conformation of acid protease was followed by CD measurements at pH 4.5. The near UV CD spectrum is shown as Fig. 3C. The enzyme exhibited peaks at 260, 266, 275, 282, 290 and 300 nm. Minima were observed at 263, 270, 279, 286 and 295 nm, corresponding to phenylalanine, tyrosine and tryptophan residues in the enzyme.

The far UV CD spectrum is shown in Fig. 3D. The spectrum shows a broad minimum in the region 213–216 nm, indicative of



**Fig. 2.** Optimum pH and temperature for acid protease activity. A. Optimum pH ( $\blacktriangle$ – $\blacktriangle$ ) and pH stability ( $\circ$ – $\circ$ ) of acid protease from *Aspergillus oryzae*. Activity measurements were carried out at 55 °C. 100 mM buffers were used: glycine–HCl (pH 2–5), acetate (pH 5–7) and glycine–NaOH (pH 7–10). B. Optimum temperature ( $\circ$ – $\circ$ ) for activity and  $T_m$  ( $\square$ – $\square$ ) for acid protease activity measurements were carried out at pH 3.2. Temperature was maintained within  $\pm 1$  °C, using a water bath.

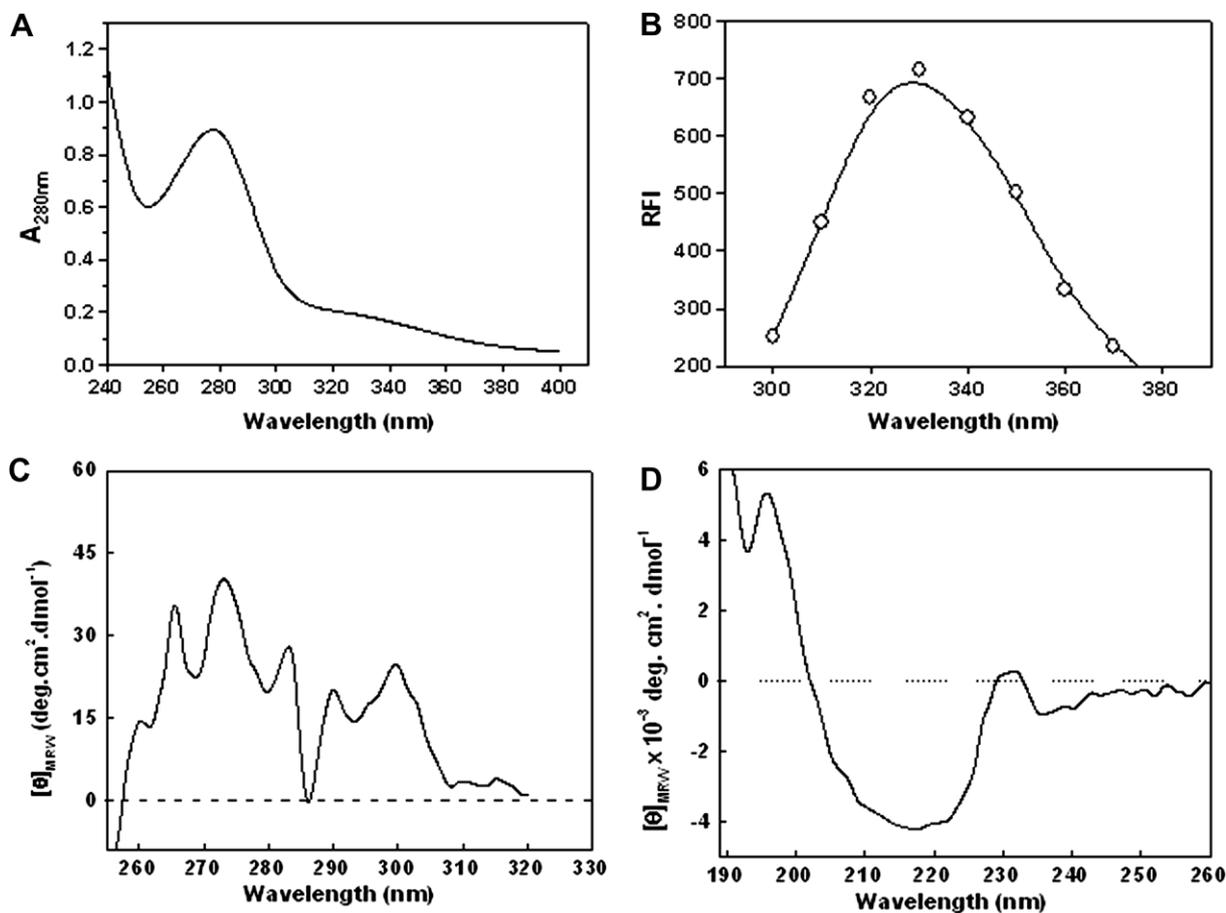
a predominant  $\beta$  structure. Two positive bands were seen at 197 and 230 nm when the spectrum was recorded at pH 4.5. The presence of these bands strongly suggested that acid protease from *A. oryzae* belongs to the family of aspartic proteases. Circular dichroism studies of this enzyme are well in accordance with other studies pertaining to acid proteases of different fungal origin (Allen, Blum, Cunningham, Tu, & Hofman, 1990; Tello-Solis & Hernandez Arana, 1995; Tello-Solis et al., 1994). The CDSSTR method, using SP175 as reference database, gave the best fit and was used to estimate secondary structure content. This method revealed 57%  $\beta$  structure, 4%  $\alpha$  helix and 38% aperiodic structure in the protein.

### 3.5. Inhibition studies

Pepstatin, a hexapeptide inhibitor, specifically inhibits acid proteases by irreversibly binding to the active site aspartates (Umezawa et al., 1970). Pepstatin (18.6  $\mu\text{M}$ ) inhibited 100% activity of acid protease while other class specific inhibitors, e.g. EDTA (10 mM), iodoacetamide (1 mM), PMSF (0.1 mM) and antipain hydrochloride (20  $\mu\text{M}$ ) did not show any inhibition. The concentra-

**Table 1**  
Purification of acid protease from *Aspergillus oryzae*

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold purity	Yield (%)
Crude	30	453	11,91,747	2630	1	100
Acetone precipitation	10	96	11,00,762	11,466	4.35	92
Ion-exchange	105	24	7,90,160	32,923	12.5	66
Gel filtration	20	8	3,49,264	43,658	16.6	29



**Fig. 3.** Spectral characterisation of acid protease from *A. oryzae*. **A.** Absorption spectra of acid protease from *Aspergillus oryzae*. The protein was prepared in 50 mM phosphate buffer, pH 6.0. **B.** Protein fluorescence was excited at 278 nm and emission was monitored from 300–400 nm at 27 °C. Protein was prepared in phosphate buffer (pH 6.0, 50 mM). **C.** Near UV CD spectra of acid protease from *Aspergillus oryzae*, 1.0 mg/ml of protein in 50 mM phosphate buffer, pH 4.5. Accumulations of three runs are shown. **D.** Far UV CD spectra of acid protease from *Aspergillus oryzae*, 0.22 mg/ml of protein in 50 mM phosphate buffer, pH 4.5. Accumulations of three runs are shown.

tions of class specific inhibitors were fixed, based on the effective concentration range of the respective inhibitor (Beynon & Salvesen, 2001). Pepstatin inhibited the enzyme competitively, with an  $IC_{50}$  value of 9.3  $\mu$ M (Fig. 4), which is similar to reported values for acid proteases (Dash, Phadtare, Deshpande, & Rao, 2001; Fusek, Lin, & Tang, 1990; Kay & Dunn, 1992; Sharma, Eapen, & Subbarao, 2005). The inhibition constant,  $K_i$ , was determined to be 0.37  $\mu$ M.

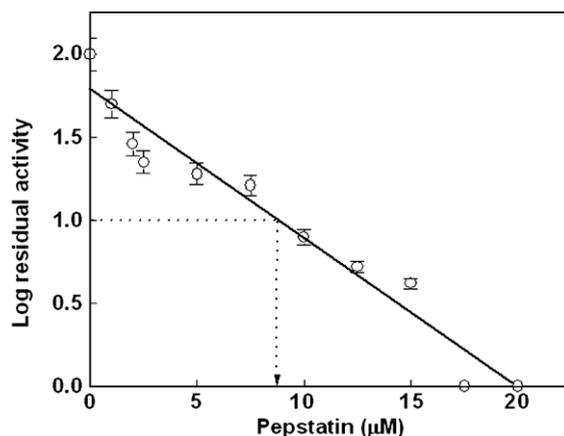
### 3.6. Activation of trypsinogen

Acid proteases possess the characteristic feature of activating native trypsinogen to active trypsin at low pH (Abita, Delaage, & Lazdunski, 1969; Davidson, Gertler, & Hofmann, 1975; Sanger & Tuppy, 1951). Acid protease from *A. oryzae* MTCC 5341 also activates trypsinogen to form trypsin, as confirmed by activity on casein (results not shown). Formation of trypsin was also confirmed by HPLC. Acid protease was found to cleave I-K residues to release active trypsin from trypsinogen.

### 3.7. Cleavage specificity

Acid proteases are endoproteolytic in nature, and show a wide range of residue specificity. Most of the acid proteases prefer bulky aromatic and hydrophobic residues at the  $S_1$  position (James, Sielecki, & Hoffmann, 1985; Lowther, Majer, & Dunn, 1995; Shintani & Ichishima, 1994; Shintani, Kobayashi, & Ichishima, 1996). Acid protease from *A. oryzae* MTCC 5341 shows endoproteolytic activity and prefers L-V and Y-T residues at the  $S_1$  and  $S_1'$  positions. This

was deduced by allowing acid protease to cleave the oxidised insulin B-chain for as least as 1 min. It was interesting to note that the same enzyme did not show any cleavage on the oxidised insulin chain A, probably due to lack of the above mentioned residue pairs in the sequence. Thus, this enzyme has cleavage specificity



**Fig. 4.** Inhibition of acid protease activity by pepstatin. **A.** Concentration of pepstatin used was in the range 0–20  $\mu$ M. Pepstatin was dissolved in alcohol (stock solution). The stock was diluted with buffer (0.05 M phosphate buffer, pH 6.0).

**Table 2**  
Properties of acid protease from *Aspergillus oryzae* MTCC 5341

Enzyme property	Acid protease
Specific activity (U/mg)	43,658
Optimum pH	3.2
Temperature optimum (°C)	55
$K_m$ (%; haemoglobin)	0.8
$V_{max}$ (μmol/min; haemoglobin)	89.4
$T_m$ (°C)	57
Molecular mass (kDa; SDS-PAGE and HPLC)	47
Cleavage specificity	L–V, Y–T and K–I

between L–V, Y–T and I–K residues. The characteristics of acid protease are summarised in Table 2.

#### 4. Conclusion

This paper reports the purification, and characterisation of one of the highest activities, of an acid protease from *A. oryzae*. The enzyme, a 47 kDa protein with a specific activity of 43,658 U/mg, belongs to the aspartate class of proteases. Acid proteases find applications in modification of food proteins and as digestive aids.

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