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Purification and characterization of an aspartic protease from the *Rhizopus oryzae* protease extract, Peptidase R

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ABSTRACT

Background: Aspartic proteases are a subfamily of endopeptidases that are useful in a variety of applications, especially in the food processing industry. Here we describe a novel aspartic protease that was purified from Peptidase R, a commercial protease preparation derived from *Rhizopus oryzae*.

Results: An aspartic protease sourced from Peptidase R was purified to homogeneity by anion exchange chromatography followed by polishing with a hydrophobic interaction chromatography column, resulting in a 3.4-fold increase in specific activity (57.5×10^3 U/mg) and 58.8% recovery. The estimated molecular weight of the purified enzyme was 39 kDa. The N-terminal sequence of the purified protein exhibited 63–75% identity to rhizopuspepsins from various *Rhizopus* species. The enzyme exhibited maximal activity at 75°C in glycine–HCl buffer, pH 3.4 with casein as the substrate. The protease was stable at 35°C for 60 min and had an observed half-life of approximately 30 min at 45°C. Enzyme activity was not significantly inhibited by chelation with ethylenediamine tetraacetic acid (EDTA), and the addition of metal ions to EDTA-treated protease did not significantly change enzyme activity, indicating that proteolysis is not metal ion-dependent. The purified enzyme was completely inactivated by the aspartic protease inhibitor Pepstatin A.

Conclusion: Based on the observed enzyme activity, inhibition profile with Pepstatin A, and sequence similarity to other rhizopuspepsins, we have classified this enzyme as an aspartic protease.

1. Introduction

Proteases, also referred to as peptidases or proteinases, are a large category of enzymes that catalyze the hydrolysis of peptide bonds. Proteases that cleave peptide bonds at the N or C termini of polypeptide chains are called exopeptidases and those that cleave peptide bonds within the polypeptide chain are classified as endopeptidases [1]. Proteases can be classified as aspartic, cysteine, glutamic, serine, and threonine proteases, depending upon the amino acids present in the active site, or as metalloproteases if a metal ion is required for catalytic activity [2]. Proteases occur naturally in all organisms and are involved in many physiological processes, including the production of nutrients for cell growth and proliferation [3], protein degradation [4], and as regulatory components for diverse physiological functions [5,6]. Microbial sourced proteases are valuable commercial enzymes that account for approximately 60% of the total worldwide sales of industrial enzymes [7]; they are used in the food, pharmaceutical, detergent, and biotechnology industries [1,8,9,10,11].

Aspartic proteases (EC 3.4.23), also known as acidic proteases, are a subfamily of endopeptidases that have been isolated from diverse sources, including viruses, bacteria, fungi, plants, and animals [12,13,14]. Several fungal aspartic proteases have been purified and characterized as rennin-like and pepsin-like enzymes [7]. The rennin-like enzymes are produced by *Endothia parasitica* (endothiapepsin, EC 3.4.23.22), *Mucor*, and *Rhizomucor* species (mucorpepsin, EC 3.4.23.23) [10]. The pepsin-like enzymes include aspergillopepsin (aspergillopepsin I, EC 3.4.23.18) from *Aspergillus* species [15] and rhizopuspepsin (EC 3.4.23.21) from *Rhizopus* species

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[16]. These enzymes have molecular weights in a range of 30–45 kDa and contain one or two conserved aspartic acid residues at the active site [2,17]. Aspartic proteases are optimally active at acidic pH (pH 3–5) and are specifically inhibited by Pepstatin A [12,15,16]. Because of their high activity and stability in acidic environments, aspartic proteases are useful reagents in the food processing industry. For example, aspartic proteases are used as milk-coagulating enzymes for the manufacture of cheese [10] and as additives to improve food flavor and texture [18].

Aspartic proteases form the dominant class of *Rhizopus* secreted proteases, known as rhizopuspepsins [3,19]. Species from which rhizopuspepsins have been isolated and biochemically characterized include *Rhizopus chinensis* [20], *Rhizopus microsporus* [21], *Rhizopus hangchow* [22], *Rhizopus oryzae* MTCC 3690 [16], and *R. oryzae* NBRC 4749 [23]. A suitable milk-coagulating enzyme must have high specific caseinolytic activity; however, many reported activity levels are poor. A neutral protease preparation derived from *R. oryzae*, Peptidase R, is used in commercial food processing to improve taste and nutritional content. Here we describe the purification and characterization of a Peptidase R-derived protein with high caseinolytic activity and an inhibition profile consistent with that of an aspartic acid protease.

2. Materials and methods

2.1. Chemicals

Peptidase R was purchased from Amano Enzyme Inc. (Nagoya, Japan). Casein, Pepstatin A, phenylmethylsulfonyl fluoride (PMSF), Pefabloc SC, N-ethylmaleimide, iodoacetic acid, β-mercaptoethanol, and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich.

2.2. Protein purification

All purification steps were performed at 4°C on an AKTA Purifier 10 system (GE Healthcare Biosciences, Uppsala, Sweden). Peptidase R (500 mg) was dissolved in 50 mL of 50 mM sodium phosphate buffer, pH 7.0, and filtered through a 25-mm Millex-HA filter (Millipore, Bedford, MA) with a 0.45-μm pore size. The enzyme solution was loaded onto a HiLoad 26/10 Q Sepharose High-Performance column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Fractions were eluted at a flow rate of 10 mL/min using equilibration buffer with a NaCl stepwise gradient of 0–0.17 M for 180 mL, 0.17–0.30 M for 480 mL, and 0.30–1.0 M for 300 mL. Fractions were detected by UV absorbance at 280 nm for collection. Aspartic protease activity in the collected fractions was measured as described in Section 2.5. Aspartic protease containing fractions were pooled, and ammonium sulfate was added to 50% (v/v) final concentration to precipitate contaminating proteins. Insoluble protein was removed by centrifugation at 12,000 rpm and 4°C for 10 min. The supernatant was applied to a HiTrap Phenyl Sepharose column (GE Healthcare Biosciences) equilibrated with 50 mM sodium phosphate buffer, pH 7.0. Aspartic protease was eluted at a flow rate of 1 mL/min using equilibration buffer with a linear descending gradient of ammonium sulfate from 100% (saturated solution) to 0%. Fractions exhibiting aspartic protease activity were combined and dialyzed against 50 mM glycine–HCl buffer, pH 3.4, for storage. The purified protein was concentrated by ultrafiltration using an Amicon Centrifugal Filter Device (10 kDa cutoff; Millipore), and stored at 4°C.

2.3. Molecular weight determination and protein quantification

Protein homogeneity and molecular weight were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 5% stacking and 12% separating gels, using broad-range protein markers (6.5–200 kDa; Bio-Rad Laboratories, Richmond, CA) as size standards. The proteins in the gel were stained with Bio-safe Coomassie Blue (Bio-Rad Laboratories). Protein molecular weight was determined using TotalLab software (Nonlinear, Durham, NC) according to the manufacturer's instructions. Protein concentrations were quantified using a Bio-Rad Protein Assay Kit with bovine serum albumin as the standard.

2.4. N-terminal amino acid sequencing

Purified enzyme was separated by SDS-PAGE gel and transferred by electroblotting to a polyvinylidene fluoride (PVDF) membrane (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer, pH 11, containing 10% methanol. The transferred protein was stained with Coomassie Blue R-250 and the band containing purified protein was excised from the membrane for N-terminal amino acid sequencing by Edman degradation using an ABI Procise 494 protein sequencer (Applied Biosystems, Foster City, CA). Comparison of the sequence with similar protease-encoding sequences in GenBank was performed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignment and identities (%) were performed using Clustal Omega on the European Bioinformatics Institute (EMBL-EBI) website (http://www.ebi.ac.uk/Tools/msa/clustalo/).

2.5. Enzyme activity assay

Proteolytic activity was measured using casein as a substrate. The reaction mixture, containing 50 μL of 2% (w/v) casein solution, 190 μL of 50 mM glycine–HCl buffer, pH 3.4, and 10 μL of the appropriate concentration of purified enzyme, was incubated at 35°C for 30 min. The reaction was terminated by adding a 250 μL of 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation at 12,000 rpm and 4°C for 10 min. The absorbance of the supernatant at 280 nm was measured using a Hitachi U3000 spectrophotometer (Hitachi, Tokyo, Japan). One unit (U) of enzyme activity was defined as the amount of enzyme required to increase the absorbance at 280 nm by 0.001 AU per minute under the aforementioned assay conditions [15].

2.6. Enzyme characterization for pH and temperature optima

The optimal pH for purified aspartic protease was determined by measuring activity at 35°C for 30 min over the pH range 2.2–4.0 using 50 mM glycine–HCl (pH 2.2–3.6) and 50 mM sodium citrate (pH 2.6–4.0) as assay buffers. The optimal temperature for protease activity was determined through assays performed using temperatures of 15–85°C for the 30 min incubation step in 50 mM glycine–HCl buffer, pH 3.4. The maximum observed activity under any of the documented reaction conditions was defined as 100% and the relative activities were calculated as a fraction of this value.

2.7. Thermostability

Thermostability of the aspartic protease was determined by incubating the purified enzyme for 65 min in 50 mM glycine–HCl buffer, pH 3.4, at 35°C and 45°C. At defined time intervals, the enzyme solutions were cooled in ice and residual activity was assayed under standard enzyme assay conditions. The relative residual activity was expressed as a percentage of the observed activity for the unheated enzyme.

2.8. Effect of chemical reagents and metal ions on enzyme activity

To determine the effects of protease inhibitors and cysteine protease activators on enzyme activity, purified enzyme was incubated with each reagent in 50 mM glycine–HCl buffer, pH 3.4, at 35°C for 30 min,

followed by measuring its activity under the standard assay conditions described above. The enzyme was assayed for inhibition by the serine protease inhibitors PMSF (0.1 mM and 1 mM) and Pefabloc SC (1 mM), the cysteine protease inhibitors N-ethylmaleimide (1 mM), and iodoacetic acid (1 mM), and the aspartic protease inhibitor pepstatin A (1 μM). Assays were also performed on the enzyme with β-mercaptoethanol (1 mM) and dithriothreitol (1 mM), which are known to enhance cysteine protease activity.

To determine whether the protease is metal-dependent, an assay was performed on the purified protein following incubation with the chelator ethylenediamine tetraacetic acid (EDTA). Specifically, purified enzyme was incubated at 35°C for 30 min in 50 mM glycine–HCl buffer, pH 3.4, with 10 mM EDTA. EDTA was removed by centrifugal ultrafiltration using an Amicon Centrifugal Filter Device (10 kDa cutoff; Millipore) prior to spectrophotometrically assaying for activity. The effect of added divalent metals on EDTA-treated enzyme was determined by incubating the enzyme following chelator removal with an aqueous metal chloride solution at a final concentration of 1 mM for 30 min. The enzyme was assayed in the presence of $CaCl_2$, $ZnCl_2$, $NiCl_2$, $CuCl_2$, $MnCl_2$, and $CoCl_2$. Relative activity is expressed as a percentage of un-treated enzyme activity.

3. Results and discussion

3.1. Purification of aspartic protease

A crude enzyme solution of Peptidase R exhibiting aspartic protease activity (17×10^3 U/mg) was separated by chromatography using a HiLoad 26/10 Q Sepharose High-Performance column and eluted with a stepwise NaCl gradient. The chromatographic profile is shown in Fig. 1a. There are two major protein peaks; the one eluting at 0.17–0.20 M NaCl contained active protein. The specific activity of the pooled active fractions was 36.7×10^3 U/mg, representing a 90.6% yield of the original enzyme activity. Active fractions were further purified by hydrophobic interaction chromatography on a HiTrap Phenyl Sepharose column where Fractions 9–15 contained target enzyme activity (Fig. 1b) and were pooled to comprise the final sample of purified protease. A summary of the purification steps for Peptidase R-derived aspartic protease is shown in Table 1. After two column chromatography purification steps, the enzyme was purified 3.4-fold from a crude Peptidase R, with a final yield of 58.8% and an observed specific activity of 57.5×10^3 U/mg. Aspartic proteases purified from *Aspergillus oryzae* MTCC 5341 [15] and *Aspergillus niger* BCRC 32720 [18] showed high proteolytic activity with a specific activity of about 43.6×10^3 U/mg and 23.3×10^3 U/mg, respectively. The activity of the aspartic protease purified here was one of the highest reported, and may be useful as a digestive aid.

Enzyme purity was confirmed by SDS-PAGE. The purified protein migrated as a single band with an apparent molecular weight of ~39 kDa (Fig. 2). The molecular weight of the purified enzyme is higher than reported for rhizopuspepsins from *R. oryzae* MTCC 3690 (34 kDa) [16], *R. oryzae* NBRC 4749 (34 kDa) [23], and *R. microsporus* (34.5 kDa) [21]. These results suggest that the purified protease from Peptidase R is a distinct, previously unreported rhizopuspepsin.

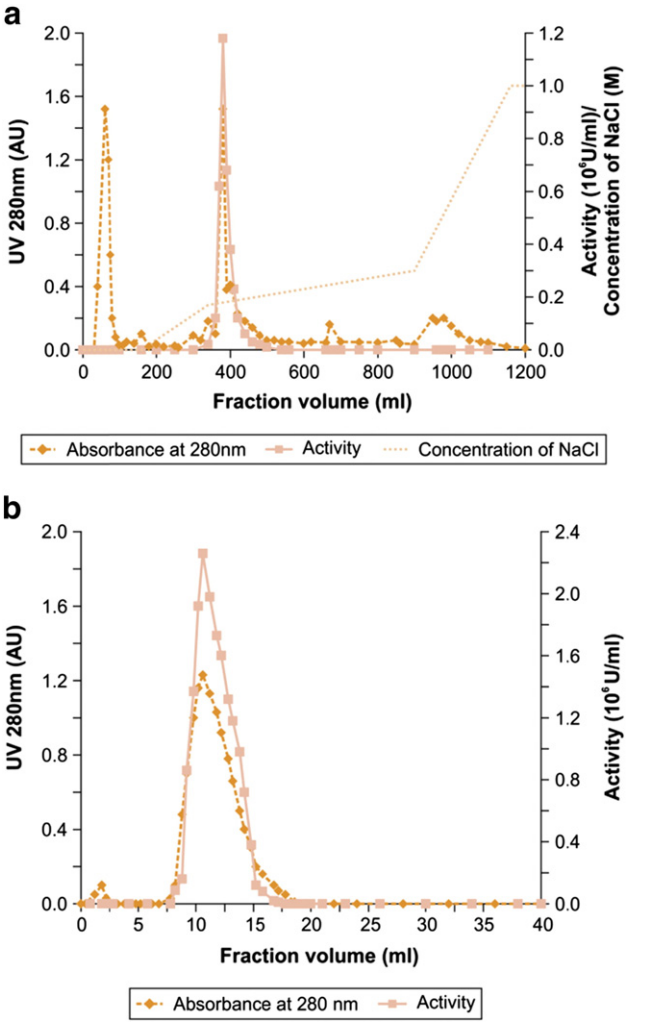

Fig. 1. Chromatographic purification of an aspartic protease from *R. oryzae* crude enzyme preparation. (a) Anion exchange chromatography using a HiLoad 26/10 Q Sepharose High-Performance column (b) hydrophobic interaction chromatography using a HiTrap Phenyl Sepharose column. Purification conditions are described in Materials and methods.

3.2. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of the purified protease was identified as Ser-Gly-Ser-Gly-Val-Val-Pro-Met-Thr-Asp-Tyr-Glu-Tyr-Asp-Ile-Glu-Tyr. Homology search and multiple sequence alignment results are shown in Fig. 3. The N-terminal amino acid sequence of the purified enzyme shows 75, 71, 69, and 63% identity to rhizopuspepsins from *R. oryzae* (GenBank accession no. ACL68093.1), *R. microsporus* var. *chinensis* (GenBank accession no. AAA33880.1), *R. niveus* (GenBank accession no. AAA33882.1), and *R. oryzae* NBRC 4749 (GenBank accession no. ACL68087.1), respectively. It was also 71% identical to syncephapepsin from *Syncephalastrum racemosum* (GenBank accession no. AAC69517.1) and was only 31% identical to aspergillopepsin I from *A. niger* (GenBank accession no.

Table 1
Purification of an aspartic protease from a *R. oryzae* crude enzyme preparation.

Purification step	Total protein (mg)	Total activity (10^6 U)	Specific activity (10^3 U/mg)	Fold purification	Yield (%)
Crude enzyme	500	8.5	17.0	1	100
Q Sepharose	210	7.7	36.7	2.2	90.6
Phenyl Sepharose	87	5.0	57.5	3.4	58.8

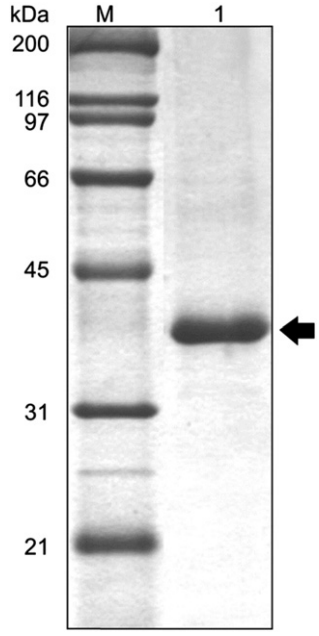

Fig. 2. SDS-PAGE profile of purified aspartic protease. Lane M, molecular weight markers; lane 1, purified aspartic protease. The arrow indicates the band corresponding to the purified aspartic protease (approximate molecular weight, 39 kDa).

XP_001401093.1). The sequence homology with rennin-like enzymes endothiapepsin and mucorpepsin was low (data not included in Fig. 3). For protein identification, the N-terminal sequence was also used to search the genome database for *R. oryzae* RA 99–880 (http://www.broadinstitute.org/annotation/genome/rhizopus_oryzae/Blast.html), but a significantly similar sequence was not identified. The N-terminal amino acid sequence of the purified enzyme showed high homology to those of rhizopuspepsins, indicating that the purified enzyme possesses aspartic protease activity.

3.3. Effects of pH and temperature on enzyme activity and stability

Protease activity of the purified enzyme was surveyed across the pH range 2.2–4 using casein as a substrate at an assay temperature of 35°C. The pH profile of the purified aspartic protease is shown in Fig. 4a. The enzyme was most active between pH values 3.0–3.6 with maximal activity at pH 3.4. Protease activity decreased significantly below pH 3.0 and was approximately 45% of the maximal activity when assayed at pH 2.2 in glycine–HCl buffer. Enzyme activity was slightly

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1  - S G S G V V P M T D Y E Y D I E Y
2  - A G V G T V P M T D Y G N D I E Y
3  - - A S G S V P M V D Y E N D V E Y
4  - - G S G S V P V T D E G N D V E Y
5  - - G V G S V P M T D Y L Y D V E Y
6  A S G T G S V P M T D V D Y D V E Y
7  - - S K G S A V T T P Q N N D E E Y
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Fig. 3. Multiple sequence alignment of N-terminal amino acid sequences of the purified and other fungal aspartic proteases. (1) aspartic protease purified from Peptidase R; (2) rhizopuspepsin from *R. microsporus* var. *chinensis* (GenBank accession no. AAA33880.1); (3) rhizopuspepsin from *R. niveus* (GenBank accession no. AAA33882.1); (4) rhizopuspepsin from *R. oryzae* NBRC 4749 (GenBank accession no. ACL68087.1); (5) rhizopuspepsin from *R. oryzae* (GenBank accession no. ACL68093.1); (6) syncephapepsin from *S. racemosum* (GenBank accession no. AAC69517.1); (7) aspergillopepsin from *A. niger* (GenBank accession no. XP_001401093.1). Identical residues are boxed.

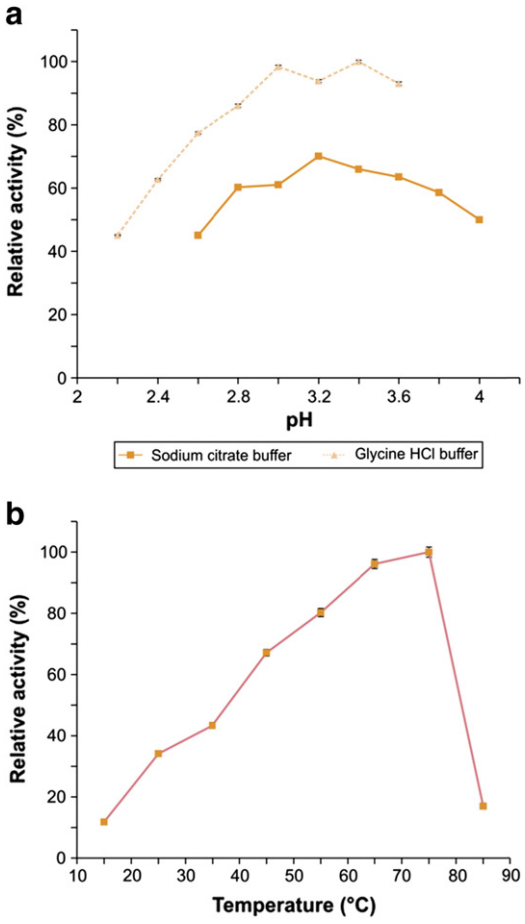

Fig. 4. Determination of the optimal pH and temperature for the purified aspartic protease. (a) The effect of pH on the activity of purified protease was determined at 35°C for a 30 min incubation, as described in Materials and methods, with either 50 mM glycine–HCl, pH 2.2–3.6 (▲) or 50 mM sodium citrate, pH 2.6–4.0 (■) buffer. (b) Temperature optima were determined in 50 mM glycine–HCl buffer, pH 3.4 at various temperatures for 30 min. The data points shown are the means for three independent experiments, and the highest observed activity was defined as 100%. The error bars indicate standard deviations.

reduced at pH 4.0 in sodium citrate buffer and was not detected at pH 7.0 (data not shown). Similar pH optima for protease activities have been reported from *R. oryzae* NBRC 4749, pH 3.0 [23]; *R. hangchow*, pH 3.0 [22]; *R. chinensis*, pH 3.1 [20]; *A. niger* I1, pH 3.0 [24]; and *A. oryzae* MTCC 5341, pH 3.2 [15]. The pH optima in the same range have also been reported for mucorpepsins from several *Mucor* species [10]. In contrast, the *R. oryzae* MTCC 3690 enzyme exhibits optimal activity at pH 5.5 [16] and the enzymes from *A. niger* BCRC 32720 [18] and *R. microsporus* exhibit optimal activity at pH 2.5 [21]. Most fungal aspartic proteases show maximal activity at pH 3–4, because the pair of aspartic acid residues in the active site must react in ionized and unionized form for catalytic activity [7]. The pH optimum might indicate a similar reaction mechanism between the purified enzyme and aspartic proteases.

During the pH optimization study, we observed that the enzyme activity was higher across pH values 2.6–3.6 in glycine–HCl versus sodium citrate buffer. This phenomenon was also observed in rhizopuspepsin from *R. oryzae* NBRC 4749, in which enzyme activity is higher at pH 3.0 in glycine–HCl buffer than in citrate phosphate buffer [23]. Glycine is a common bulking agent in pharmaceutical protein formulations and is used to stabilize the protein during freeze–thawing solution [25]. Glycine has also been used to improve the stability of alkaline proteases at high temperature and in detergents [26]. We suggest that glycine is protonated at low pH in the glycine–HCl buffer; this might

prevent protein aggregation in the buffer system, leading to higher proteolytic activity. The effects of glycine on the aggregation and stability of the purified aspartic protease will be examined in the future.

As shown in Fig. 4b, hydrolytic activity of the purified protease increased with increasing temperature, reaching a maximum at 75°C, which is higher than those previously reported for rhizopuspepsins from *R. oryzae* NBRC 4749 (50°C) [23] and *R. oryzae* MTCC 3690 (60°C) [16]. The thermostability profile of the purified protease is shown in Fig. 5. At 35°C, the enzyme retained full activity after 1 h of incubation. At 45°C, enzyme activity decreased with increasing incubation time; the observed half-life for the protease was approximately 30 min at 45°C. Rhizopuspepsin from *R. oryzae* MTCC 3690 has a half-life of approximately 20 min at 60°C and retains full activity after incubation at 40°C for 1 h [16]. Moreover, half-lives of approximately 3.5 h and 10 min at 40°C and 60°C, respectively, were reported for rhizopuspepsin 6 from *R. oryzae* NBRC 4749 [23]. These results suggest that aspartic proteases from *Rhizopus* species seem to be stable at temperatures below 40°C and prone to inactivation above this temperature.

3.4. Effects of inhibitors and metal ions

To characterize the purified protease, the effects of various inhibitors on enzyme activity were examined. The purified protease was strongly inhibited by Pepstatin A, a hexapeptide inhibitor that specifically and irreversibly binds aspartate within the aspartic protease active site, with complete inactivation occurring at an inhibitor concentration of 1 μM (Table 2). In contrast, serine protease inhibitors (PMSF and Pefabloc SC) and cysteine protease inhibitors (*N*-ethylmaleimide and iodoacetic acid) did not inhibit enzyme activity even at 1 mM (data not shown). The cysteine protease activators dithiothreitol (1 mM) and β-mercaptoethanol (1 mM) did not increase protease activity (data not shown). Strong inhibition by the aspartic protease inhibitor, Pepstatin A, suggests that the purified protein may be classified as an aspartic protease.

The effects of EDTA and metal ions on EDTA-treated enzyme were examined at pH 3.4 and 35°C using casein as substrate (Table 2). The chelating agent EDTA (10 mM) had no effect on enzyme activity,

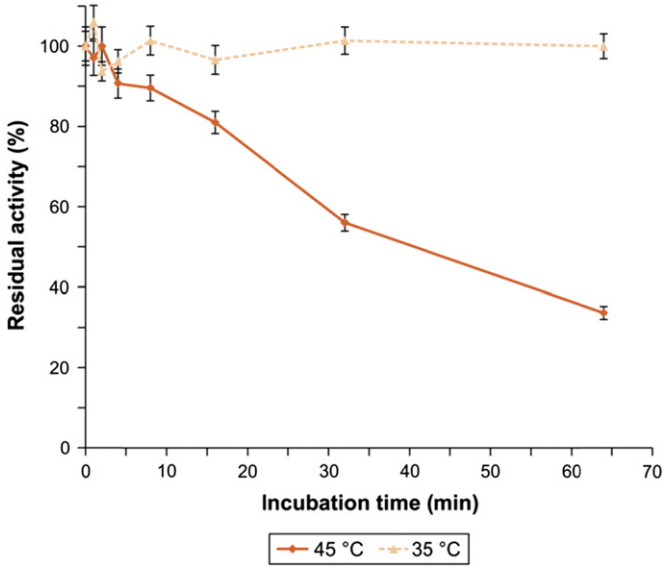

Fig. 5. Determination of thermostability of the purified aspartic protease. Thermostability was determined through assays of purified enzyme in 50 mM glycine–HCl buffer (pH 3.4) incubated at 35°C (▲) or 45°C (◆) and enzyme activity was assayed under standard conditions. The residual activity is expressed as the percentage compared to the activity of un-incubated enzyme. The data points shown are the means of three independent experiments and the error bars indicate standard deviations.

Table 2
Effects of protease inhibitors and various metal ions on purified enzyme activity.

Compound	Concentration	Relative activity (%)[a]
None	0	100.0 ± 3.6
Pepstatin A	1 μM	0
EDTA	10 mM	101.4 ± 3.8
$CaCl_2$	1 mM	98.6 ± 3.5
$ZnCl_2$	1 mM	87.8 ± 3.2
$NiCl_2$	1 mM	84.2 ± 2.9
$CuCl_2$	1 mM	87.2 ± 2.6
$MnCl_2$	1 mM	84.7 ± 2.3
$CoCl_2$	1 mM	88.3 ± 2.7

[a] Enzyme activity assayed in the absence of metal ions and inhibitors was defined as 100% activity.

suggesting that it is not a metalloprotease. The addition of 1 mM Ca^{2+} had no effect on proteolytic activity, while the addition of 1 mM Zn^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, and Co^{2+} slightly reduced enzyme activity. These results indicate that the activity of the purified enzyme is not metallo-dependent. Metal ions have varying effects on the activity of aspartic proteases from different fungi. Rhizopuspepsin from *R. chinensis* is significantly inhibited by Fe^{3+}, while no inhibitory effect is observed with Cu^{2+}, Hg^{2+}, Fe^{2+}, and Pb^{2+} [20]. Fe^{2+} strongly activates and Fe^{3+}, Cr^{3+}, Sb^{3+}, Pb^{2+}, Sn^{2+}, Sr^{2+}, and Ag^{+} strongly inhibits the enzyme from *A. niger* BCRC 32720 [18]. Cu^{2+} strongly inhibits the enzyme from *A. niger* I1 [24]. Ca^{2+} is a potent activator that combines with para-κ-casein to form firm clots during milk clotting [10]. The proteolytic activity of purified aspartic protease was not inhibited by Ca^{2+}, supporting its potential utility in food processing.

4. Concluding remarks

An aspartic protease was purified to homogeneity from a commercial Peptidase R preparation by liquid chromatography using a strong anion exchange column followed by a hydrophobic interaction chromatography step, resulting in a 3.4-fold increase in specific activity and a yield of 58.8%. The molecular weight of the purified enzyme was determined to be approximately 39 kDa by SDS-PAGE. The purified enzyme appears to be an aspartic protease, based on observed inhibition in the presence of Pepstatin A. The enzyme was optimally active at pH 3.4 and stable at 35°C, appropriate for biotechnological applications and food processing. Genetic and protein structural analyses are in progress to elucidate the biochemical properties of and potential industrial applications for this enzyme.

Conflict of interest

The authors declare that they have no conflict of interets.

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