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Partial Characterization of an Acidic Protease from *Rhizopus stolonifer* RN-11

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Abstract: The present study characterises an acidic protease purified from the *Rhizopus stolonifer* strain, RN-11. The acidic protease with a 70 kDa molecular weight, was stable within pH 2-4 at temperatures 40-50℃. The temperature and pH value conducive to optimal catalytic activity were pH 2.5 and 50℃, respectively. Treatment with 5 mmol metal ions showed that the acidic protease was activated by Na^+, K^+, Mn^{2+}, Cu^{2+} and Ca^{2+}, inhibited by Zn^{2+}, Li^{2+} and Fe^{2+}, and unaffected by Mg^{2+}. It was proposed that the studied acidic protease might represent a previously uncharacterised type of acidic protease produced by the *Rhizopus stolonifer* RN-11 strain.

Keywords: Enzyme characteristics, purification, Rhizopuspepsins.

1. INTRODUCTION

Acidic proteases degrade proteins in acidic conditions, rendering them useful for medical applications, food processing, and animal feed production [1]. Several studies have isolated acidic proteases from different fungi, including *Aspergillus niger* [2, 3], *Aspergillus oryzae* [4-6], *Penicillium* spp. [7, 8], *Mucor pusillus* [7, 9], *Candida albicans* [10], and *Rhizopus* spp. [11]. Rhizopuspepsins are a family of acidic proteases isolated form the *Rhizopus* genus [12, 13]. Rhizopuspepsins have been isolated and biochemically characterized in *R. chinensis* [14], *R. microsporus* [15], *R. hangchow* [16], *R. oryzae* MTCC 3690 [11], and *R. oryzae* NBRC 4749 [17]. However, few studies concern the characterisation of *Rhizopus stolonifer* isolates. The present study characterises an acidic protease purified from the *Rhizopus stolonifer* strain RN-11.

2. MATERIAL AND METHODS

Strain screening and culture conditions: The strains used in this study were provided by the Industry Culture Collection Center of Henan University of Technology. *Rhizopus stolonifer* was activated and grown on seed medium (1:10 (w:v)) with a bran-water ratio, boiled for 30 minutes, and then filtered through four layers of gauze with 0.5% (w) ammonium sulphate for 48 hours at 30°C with gentle agitation. 100 μL of filtered fermentation broth was added into sample wells containing separation medium (Czapek medium plus 1% casein (w), pH 3-5) and incubated at 50°C overnight. The RN-11 *Rhizopus stolonifer* strain was selected for its conspicuous acidic protease activity.

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ITS sequence analysis and morphology: 48 hour cultures grown on seed medium at 30°C with gentle agitation were used for Genomic DNA (gDNA) isolation. gDNA was extracted from cultures using the SDS-CTAB method [18]. The primers used for ITS sequence amplification were ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). PCR programs were run with a Biometra Tpersonal-48 PCR Thermal Cycler, consisting of an initial denaturation step (95°C for 5 minutes) followed by 30 cycles comprising of denaturation (94°C for 30 seconds), annealing (55°C for 1 minute), extension (72°C for 1 minute), and a final extension step (72°C for 10 minutes). Fragments were purified from agarose gel using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shang Hai, China) and entrusted to Life Technologies (Shang Hai, China) for ITS sequencing. Strain morphology was observed and identified using the methods described by Wei [19].

Protein determination: Protein concentration of purified acidic protease was determined with the Bradford method [20].

Purification of acidic protease: The RN-11 strain was inoculated into 100 mL seed medium and incubated at 30°C with gentle agitation for 48 hours. Culture supernatant was diluted to 10^7CFU/mL spore suspension. 1mL spore suspension was inoculated into the solid fermentation medium(15 g bran, 10.7 mL water, 0.69 g sucrose and 1.0 g, $NaNO_3$, K_2HPO4 0.042 g, pH 2.5), and incubated at 30°C for 72 hours. The crude enzyme solution was extracted from pulverized solid fermentation products with lactic acid buffer (30:1 (v:w) liquid-solid ratio, pH 3.0) at 30°C for 105 minutes, and centrifuged at 4°C with 4200g. Ammonium sulfate (w) was added to 100 mL crude enzyme solution to achieve 50% saturation, thereby removing impure proteins. The enzyme solution was incubated at 4°C for 24 hours, centrifuged at 8200g for 10 minutes, and then the precipitate

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Table 1. Purification of Proteases from *Rhizopus stolonifer* RN-11.

Procedure	Total activity (U)	Total protein (mg)	Specific activity （U/mg）	Fold purification	Yield (%)
Crude enzyme	80120.00	4493.60	17.83	1.00	100
$(NH_4)_2SO_4$ fraction	69960.78	2713.76	25.78	1.45	87.32
Dialysis	56773.03	104.95	540.96	30.34	70.86
Sephadex G-75 fraction	19541.27	26.23	745.00	41.78	24.39

was discarded. Ammonium sulfate (w) was then added to the supernatant to achieve 80% saturation, incubated at 4°C for 24 hours, and centrifuged at 8200g for 10 minutes, whereupon the precipitate was resuspended in 15 mL lactate buffer (pH 3.0). This enzyme solution was lyophilized and resuspended in 8 mL lactate buffer (pH 3.0) post dialysis. The resulting sample was loaded onto Sephadex G-75 medium, which was equilibrated with citrate (pH 5.0) and then eluted with the same buffer at a flow rate of 3-6 mL/minute. Fractions with acidic protease activity were lyophilized and subjected to the following assays.

Enzyme activity assay: Enzyme activity was determined by the conventional folin phenol method as described by Lowry *et al.* [21]. One unit of enzyme activity was registered upon the release of 1 μg of tyrosine - resulting from the hydrolysis of casein - within 1 minute at 50°C and pH 2.5.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE): The purified enzyme was dissolved in distilled water. 15 μL of sample and 15 μL of standard protein were mixed, loaded onto a 12.5% acrylamide gel, and resolved with SDS-PAGE according to the method described by Weber and Osborn [22]. Gels were stained with Coomassie Brilliant Blue R-250, and the molecular weight of the acidic protease was determined relative to the standards.

Effects of pH and temperature on acidic protease activity and stability: Enzyme and substrate were suspended in serial lactate buffer dilutions (pH 1.5-5.5), mixed, and incubated at 40°C for 10 minutes, whereupon the enzyme activity was measured. Enzyme activity at various pH values was calculated relative to the highest enzyme activity. Subsequently, the serial pH enzyme solutions were incubated at room temperature for 24 hours, and the residual activity and pH stable range of enzyme were determined. To ascertain the effect of temperature on enzyme activity, the enzyme and substrate were suspended in lactate buffer (pH 2.5), and the activity of acidic protease was measured at various temperatures (30-70°C) relative to the highest enzyme activity. To measure thermostability, the acidic protease-lactate buffer suspension was incubated at various temperatures (30-70°C) for various durations (30-120 minutes). At discreet time intervals, the enzyme solution was cooled down immediately for 5 minutes in ice-cold water. Enzyme activity was calculated relative to the activity recorded following two minutes incubation at each temperature (30-70°C).

Effect of metal ions: Various metals (Na^+, K^+, Mn^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Fe^{2+}, Cu^{2+}, and Li^{2+}) were suspended in lactate buffer (pH 2.5), and were added to the purified acidic protease for a final concentration of 5 mM. The samples were allowed to stand for 30 minutes and subsequently incubated for 10 minutes at 50°C, whereupon the relative residual activity was measured.

3. RESULTS AND DISSCUSION

ITS analysis and morphology: ITS sequence of RN-11 strain was deposited in GenBank (accession No. HM212636). The RN-11 strain had white thick mycelium with black sporangium that had spread to the entire surface of the medium to form large colonies. The RN-11 strain was identified as *Rhizopus stolonifer* by manual and molecular identification.

Purification of acidic protease: The acidic protease was purified for electrophoretical homogeneity by ammonium sulfate precipitation, dialysis, and Sephadex G-75 gel filtration. The acidic protease was purified 41.8-fold and the recovery yielded 24.4% after Sephadex G-75 chromatography (Table **1**). Acidic proteases of varying molecular weights were previously isolated from bacteria, including an aspartate protease from *Phycomyces blakesleeanu* (35 kDa) [23], aspartate protease from *Rhizopus oryza*e (34 kDa [11] and 47.5 kDa [24] variants), protease A(37 kDa) and protease B (34 kDa) from *Aspergillus niger* [25], Aspartic protease Sapt1p (44 kDa), Sapt2p (49 kDa), Sapt3 (55 kDa proproitein and 48 kDa mature protein) [10], and two forms of acid protease (M1 150 kDa and M2 60 kDa) from *Aspergillus oryzae* [26]. Unlike previously reported data, the purified acidic protease in this study was a monomer with an MW of ~70 kDa according to SDS-PAGE analysis (Fig. **1**). The greater MW observed in the present study may be species-specific.

Effects of pH and temperature: The purified protease was stable at pH 2.0-3.0 and exhibited maximal activity at pH 2.5 (Fig. **2**). Furthermore, ≥70% activity remained after 24 hours of incubation at room temperature at pH ranges of 2.0-4.0, in conformity with the results reported by Li [27], Jose [23], Eneyslaya [28], Yin [24], and Chen [25]. According to the studies conducted by Hsiao [29], Kumar [11], Li [33] and Xu [35], the optimal pH values of extracellular acid protease from *Rhizopus oryzae*, and *Aspergillus* spp. were 3.4, 5.5, 3.5, and 4.0, respectively. From the data obtained in this study, the optimal pH for the hydrolysis of casein was 2.5 (Fig. **3**), which was markedly lower than that of acidic proteases reported elsewhere.

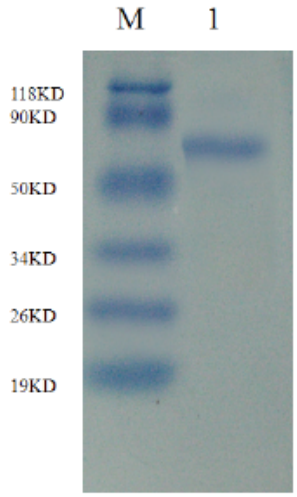

Fig. (1). SDS-PAGE of purified acidic protease from *R. stolonifer* RN-11 strain. The enzyme was resolved on a 12.5% acrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane M, molecular weight markers; lane 1, purified acidic protease (~ 70 kDa molecular weight).

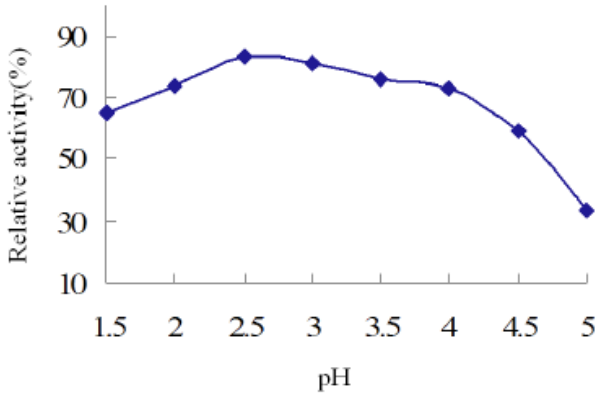

Fig. (2). pH stability of the purified *R. stolonifer* RN-11 acidic protease. The relative activity was expressed as percentage relative to the highest activity (100%).

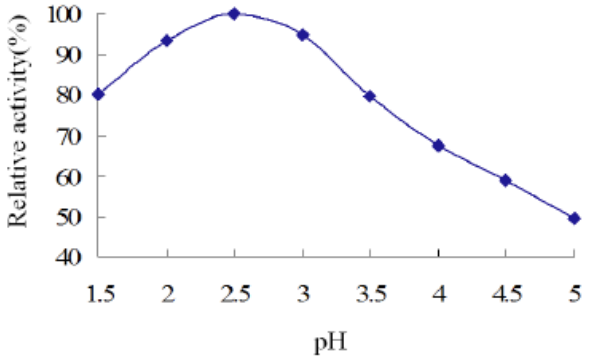

Fig. (3). Optimum pH of the purified *R. stolonifer* RN-11 acidic protease. The relative activity was expressed as percentage relative to the highest activity (100%).

The purified acidic protease in the present study exhibited certain activity at various temperatures (30-70°C). Relative activity within the range of 30-50°C increased from 60% to 100% with an increase in temperature, but rapidly declined with increasing temperature to 38.9% of its maximal at 70°C (Fig. **5**). The optimum temperature for acid protease activity was 50°C (Figs. **4** and **5**). This was similar to the rhizopuspepsin from *Rhizopus oryzae* NBRC 4749 [25] and acid protease from *Aspergillus foetidus* [34], but lower than that of rhizopuspepsins (60°C) from *R. chinensis* Saito [14] and *R. oryzae* MTCC 3690 [11].

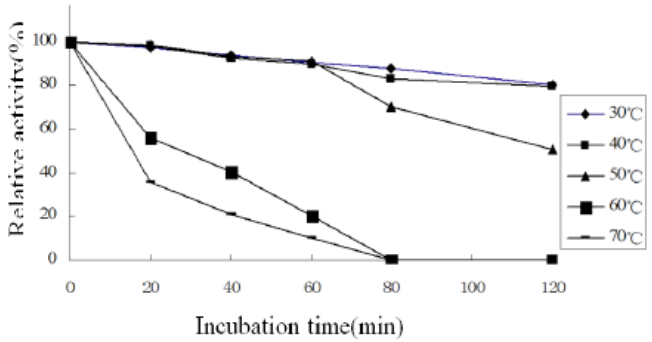

Fig. (4). Thermostability of the purified *R. stolonifer* RN-11 acidic protease. The relative activity was expressed as percentage relative to the highest activity (100%).

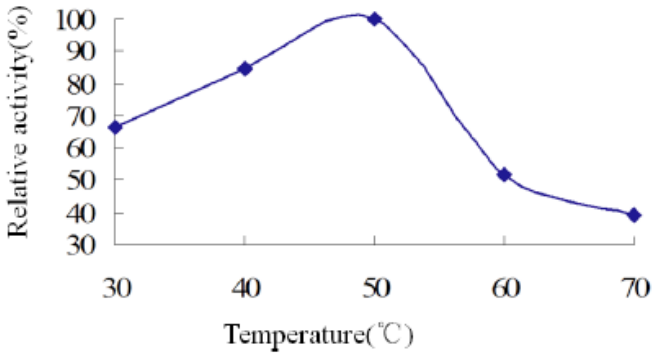

Fig. (5). Optimum temperature of the purified *R. stolonifer* RN-11 acidic protease. The relative activity was expressed as percentage relative to the highest activity (100%).

The purified acidic protease was stable within the range of 30-50°C, but unstable at temperatures >50°C (Fig. **5**). At 30-40°C, the enzyme showed maximal activity at >90% after 120 minutes of incubation. At 50°C, enzyme activity decreased slowly to ~90% after 60 minutes of incubation; at 60°C and 70°C, enzyme activity declined sharply to 55.7% and 35.6% after only 20 minutes of incubation, respectively. The observed half-life for the protease was approximately 120 minutes at 50°C. The thermostability of acidic proteases highly varied within the *Rhizopus oryzae* (30-45°C) [11], *Aspergillus oryzae* MTCC 5341 (40-57°C) [30], *A. niger* I1 (30-40°C) [3], and *Mucor pilosus* (25-55°C) [31] species. Rhizopuspepsin from *R. oryzae* MTCC 3690 had a half-life of ~20 minutes at 60°C, and retained full activity after

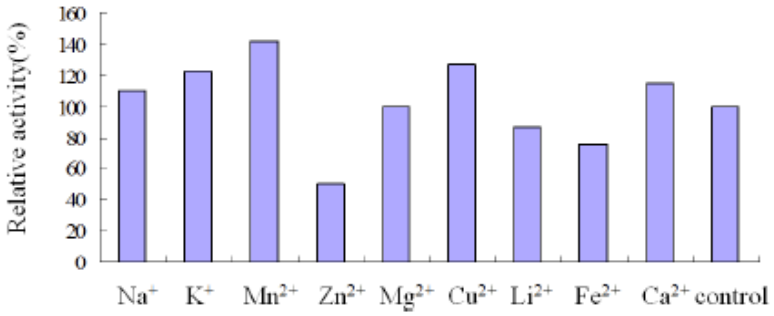

Fig. (6). Effect of metal ion on *R. stolonifer* RN-11 acidic protease activity. The relative activity was expressed as percentage relative to the control (activity of acidic protease in the absence of metal ions).

incubation at 40°C for 1 hour [11]. Moreover, Chen *et al.* reported that rhizopuspepsin 6 from *R. oryzae* NBRC 4749 had half-lives of 210 and 10 minutes following incubation at 40 and 60°C, respectively, [17]. These results, in conformity with the results of the study conducted by Hsiao *et al.* [29], suggest that aspartic proteases from *Rhizopus* species are stable at temperatures below 50°C and are prone to inactivation at greater temperatures.

Effect of metal ions: The addition of 5mM Na^+, K^+, Mn^2, Cu^{2+}, or Ca^{2+} improved enzyme activity <10%, whereas $Mn2^+$ improved enzyme activity by 40%. The addition of 5mM Mg^{2+} had no effect on proteolytic activity, while the addition of 5mM Zn^{2+}, Li^{2+}, or Fe^{2+} reduced enzyme activity (Fig. **6**). Ca^{2+} was a potent activator [29] [32], causing a 2.5-fold increase in milk clotting activity compared to a negative control. Mg^{2+} and Mn^{2+} also had stimulatory effects [11], which were also observed in the present study. Some metal ions seemed to have various effects on the activity of acidic proteases from different fungi. Rhizopuspepsin from *Rhizopus chinensis*, for example, was strongly inhibited by Fe^{3+}, but unaffected by Cu^{2+}, Hg^{2+}, Fe^{2+}, and Pb^{2+} [14]. Meanwhile, Fe^{2+} is a potent activator of the acidic protease from *Aspergillus niger* BCRC 32720, whereas Fe^{3+}, Cr^{3+}, Sb^{3+}, Pb^{2+}, Sn^{2+}, Sr^{2+}, and Ag^+ were potent inhibitors [24]. Cu^{2+} strongly inhibited the acidic protease from *A. niger* [3].

In conclusion, the acid protease isolated from *Rhizopus stolonifera* RN-11 was purified with a 24.4% recovery yield, and was characterised with an optimal pH of 2.5 and was stable below 50℃.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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