

Partial Characterization of Purified Protease Produced from *Rhizopus oligosporus* Using a By-Product of Oil Industry

¹Hafiz Muhammad Nasir Iqbal, ¹Ishtiaq Ahmed and ²Muhammad Asif Khan

¹Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad, Pakistan

²National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan

Abstract: The present study was carried out to purify and characterize an acidic protease produced from *Rhizopus oligosporus* using sunflower meal a by-product of oil industry. *Rhizopus oligosporus* cultured under some previously optimized solid state fermentation conditions. Maximum activity of 195±2.36U/mL achieved when sunflower meal was inoculated with 1% inoculum size and 20% substrate concentration at pH 3 for 72 h fermentation time period. A purification fold of 4.11 with specific activity and % recovery of 129U/mg and 9.93% was achieved respectively after ammonium sulfate precipitation and DEAE Cellulose column chromatographic technique. Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) showed purified protease has a relative low molecular weight of 43kDa. The enzyme was completely active at 4 and 50°C as an optimum pH and temperature respectively. Using casein as substrate, the enzyme showed maximum activity (V_{max}) of 158U/mL with its corresponding K_M value of 57 μ M.

Key words: *Rhizopus oligosporus* • Acidic protease • SDS-PAGE • Characterization

INTRODUCTION

Proteases are protein-digesting enzymes that are chiefly classified on the source of their optimum pH as acidic, neutral and alkaline proteases. Proteases account for approximately 60 % of all worldwide sales [1], because they have diverse applications in a wide variety of industries, such as in detergent, pharmaceutical, leather, the food industry, cheese making industry and the waste processing industry [2-4].

A broad range of micro-organisms are an excellent source of enzymes owing to their extensive biochemical diversity [5], mode of action and specificity and their susceptibility to genetic manipulation has attracted worldwide attention in attempts to exploit their physiological and biotechnological application [6,7]. Fungal species e.g. *Rhizopus oligosporus* IHS13, *Aspergillus niger*, *Rhizopus oryzae* and *Conidiobolus* spp [6-9] have ability to elaborate a wider variety of proteases enzymes other than bacteria [1, 10]. *Rhizopus oligosporus* produces protease, has a high proteolytic activity in the Tempe fermentation and further more, does not produce toxins [11]. The fungal proteases are active over an extensive pH and temperature range and

exhibit broad substrate specificity and can be suitably produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH=2.5 and 6.0 [1].

In this paper we aimed to purify and characterize an acidic protease produced from *Rhizopus oligosporus* to present potential and possible application for industrial purposes.

MATERIALS AND METHODS

Chemicals and Substrate: All the chemicals were of analytical grade and used as such. Sunflower meal a by-product of oil industry was used as growth supported substrate.

Micro-Organism and Inoculum Development: Pure culture of *Rhizopus oligosporus* was grown on potato dextrose agar slants at 30°C for 3-5 days and stored at 4°C for subsequent experimental work. The inoculum was prepared by transferring loopful culture of *Rhizopus oligosporus* into 100mL of inoculum medium contained, 2.0 g NH_4SO_4 , 0.3g K_2HPO_4 , 0.5g NaCl, 0.5g MgSO_4 , 0.2g Na_2HPO_4 and 0.1g CaCl_2 and sterilized. The inoculated

medium was incubated in a water bath shaker at 140 rpm to obtain homogeneous spore suspension up to $1 \times 10^{6.8}$ cells/mL.

Production and Isolation of Acidic Protease: An acidic protease from *Rhizopus oligosporus* was produced under some pre-optimized fermentation conditions i.e. (1% of inoculum size, 20% substrate concentration at pH=3 for 72 h of fermentation time period). From the fermented samples enzyme was isolated by adding 100mL distilled water and kept in a rotary shaker at 140rpm for half an hour to homogenize the fermented flasks. Then the fermented broth was centrifuged at $9000 \times g$ for 10 min at 4°C to get clear supernatant containing enzyme solution and the resultant clear supernatant was used for protease assay and purification purposes.

Proteolytic Activity and Protein Contents: The enzyme assay was performed by the method of McDonald and Chen [12] as described earlier [13]. The amount of protein contents in the crude enzyme extract was estimated by the method of Bradford [14] using Bovine serum albumin as standard.

Purification Process: Crude extract of protease obtained from *Rhizopus oligosporus* was centrifuged at $10,000 \times g$ for 30 min at 4°C . After centrifugation, solid crystals of ammonium sulfate were added slowly to the crude extract with constant stirring till 70% saturation was obtained. The pellets recovered by centrifugation at $10,000 \times g$ for 30 min were suspended in 50mM phosphate buffer, pH=6.0 and dialyzed against repeated changes of the same buffer to remove extra crystals of ammonium sulfate. Total protein and activity of partially purified protease were determined before and after dialysis of ammonium sulfate precipitation as mentioned before.

Ion Exchange Chromatography: To attain further purification dialyzed extract was subjected to ion exchange chromatography using DEAE cellulose column (2×20 cm) equilibrated with 0.5 M potassium phosphate buffer (pH=6.5). A total of 25 fractions of 2mL each were collected at a flow rate of 1 mL/2min and both the enzyme activity and protein contents were determined for each separate fraction, as mentioned in the previous section.

SDS-PAGE: Sodium dodecyl sulfate poly acryl amide gel electrophoresis (SDS-PAGE) was performed on a 5 % stacking and a 12 % running gel according to the

method of Laemmli [15] to determine the molecular weight of purified protease. The molecular weight of the purified protease was determined in comparison with standard molecular weight markers.

Characterization of Purified Acidic Protease: The purified protease fractions obtained from DEAE-cellulose column were subjected to characterization through kinetic studies by studying the effect of different pH values (1-7), effect of different incubation temperatures ($30-60^{\circ}\text{C}$) and effect of varying concentrations of substrate on purified acidic protease produced from *Rhizopus oligosporus*. The enzyme activities for each case were determined under standard assay conditions using casein as substrate.

RESULTS AND DISCUSSION

Production of Acidic Protease: *Rhizopus oligosporus* was cultured in fermentation medium containing sunflower meal as growth supported substrate under optimum fermentation conditions and maximum acidic protease activity of $195 \pm 2.36 \text{ U/mL}$ obtained when sunflower meal was inoculated with 1% inoculum size, 20% substrate concentration at pH=3 for 72 h fermentation time period [13]. A low cost substrate like sunflower meal and its low concentration is effective for growth and enzyme production [16, 17].

Purification and SDS-PAGE: The crude enzyme was precipitated at 70 % saturation with specific activity of 46.4 U/mg and 1.48 fold purification. After column chromatography the enzyme was purified up to 4.11 fold with a yield of 9.93 % and specific activity of 129U/mg (Table 1). The purified acidic protease was found to be a homogenous monomeric protein as evident by a single band corresponding to 43kDa on SDS-PAGE (Fig. 1). Most of the halophilic and acidic proteases reported as a single band and have molecular weight in range from 40 to 130kDa [18, 19]. The present reported protease of *Rhizopus oligosporus* was evidently different, in molecular weight (43kDa), from other reported fungal proteases like from *Rhizopus oryzae* (31kDa) [20], *M. purpureus* CCRC31499 (40kDa) [21], *Synergistes* sp. (60kDa) [5] and *Aspergillus fumigatus* (124kDa) [22].

Characterization of Purified Acidic Protease

Effect of pH on Protease Activity: Results of enzyme assay showed that the acidic protease was completely stable in a large pH range (3-5) and presented an optimum

Table 1: Purification summary of acidic protease

| Purification Steps | Total Volume (mL) | Total Enzyme Activity (U) | Total Protein Content (mg) | Specific Activity (U/mg) | Purification fold | % Yield |
|---|-------------------|---------------------------|----------------------------|--------------------------|-------------------|---------|
| Crude Enzyme | 100 | 19500 | 622 | 31.4 | 1.0 | 100.00 |
| (NH ₄) ₂ SO ₄ | | | | | | |
| Precipitation | 22 | 4730 | 102 | 46.4 | 1.48 | 24.30 |
| Dialysis | 18 | 4140 | 62 | 66.8 | 2.13 | 21.20 |
| DEAE Cellulose | 8 | 1936 | 15 | 129.0 | 4.11 | 9.93 |

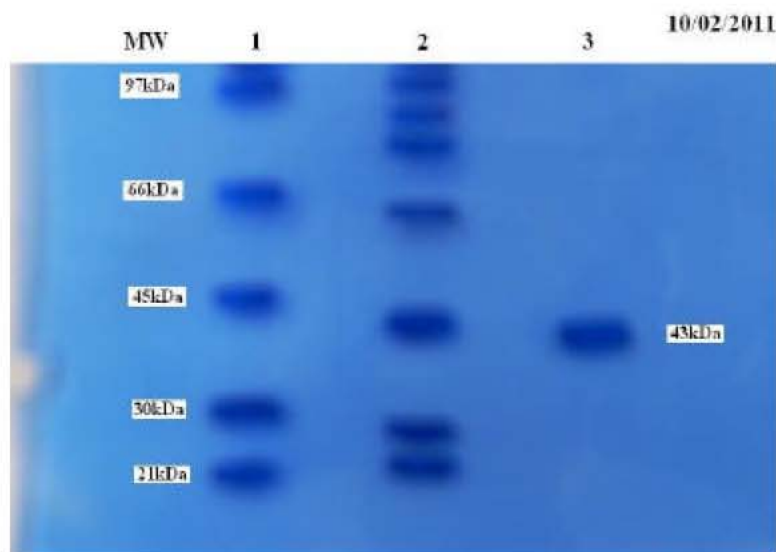


Fig. 1: Determination of molecular weight of an acidic protease by SDS-PAGE

[Lane MW, Molecular weights in kDa; lane 1, standard protein markers (Phosphorylase B, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa and trypsin inhibitor, 21 kDa); lane 2, Crude enzyme extract; lane 3, Purified acidic protease (43 kDa)]

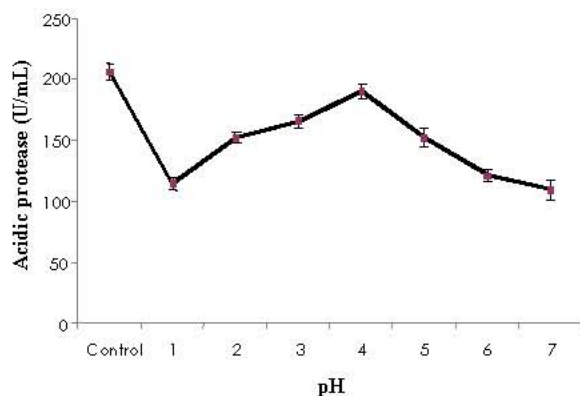


Fig. 2: Effect of varying pH values on an acidic protease activity

activity 190U/mL at a pH=4 (Fig. 2) whereas any further increase in pH up to 7 showed decreasing trend in activity. Kumar *et al.* [20] also reported the pH optima in the same pH range of 5.5-6.5 for *R. oryzae* with optimum at 5.5. The pH optima for acidic protease from other fungal sp has been reported to be vary from 2.5-6.0 [1].

Effect of Temperature on Protease Activity: To investigate the thermal stability of protease, enzyme was incubated at different temperatures ranging from 30-60°C. Temperature optimum for purified acidic protease was observed at 50°C. Results of figure 3 showed that at temperature higher than 50°C enzyme starts to losses its activity rapidly. For a variety of industrial and biotechnological applications thermo stability is an attractive and advantageous characteristic of an enzyme [23-25]. Hussain *et al.* [26] reported maximum proteolytic activity at 40°C while, further increase decrease the activity and showing 80% loss in activity at 70°C. The present reported protease from *Rhizopus oligosporus* was heat stable with optimum in activity at 50°C and different from other reported proteases like from *Aspergillus niger* at (45°C) [27], *Aspergillus niger* (32°C) [28] and *P. roqueforti* P2 (25°C) [29].

Determination of K_M and V_{max} : The kinetic constants K_M and V_{max} were determined for purified acidic protease using varying concentration of casein ranging from

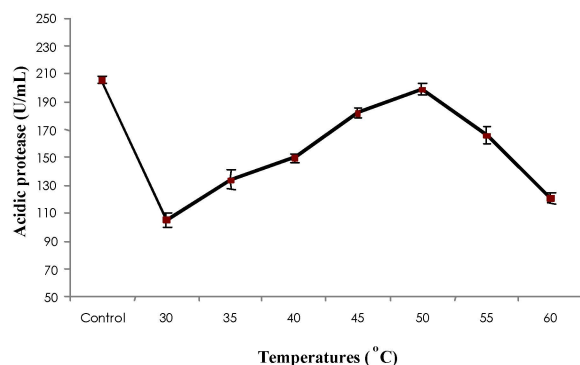


Fig. 3: Effect of different temperatures on an acidic protease activity

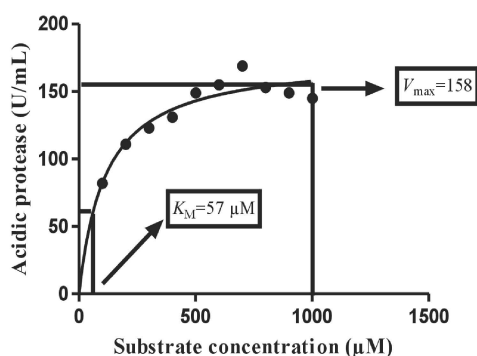


Fig. 4: Determination of K_M and V_{max} for purified acidic protease through Michaelis-Menten kinetics

100-1000 μM . Results obtained were plotted as a graph of Enzyme activity (U/mL) against concentration of substrate (μM), which yielded a hyperbolic curve, as shown in the figure 4. The catalytic properties K_M and V_{max} values of purified acidic protease from *Rhizopus oligosporus* were $58\mu\text{M}$ and 148U/mL respectively. An acidic protease is highly substrate specific and exhibit maximum activity towards casein as substrate [30, 31]. Adinarayana *et al.* [32] reported that protease have a high level of hydrolytic activity against casein as substrate and poor to moderate hydrolysis of BSA and egg albumin respectively. Patel *et al.* [33] reported the K_M and V_{max} of Ve1 protease were 0.153 g/100mL and 454U/mL respectively.

CONCLUSION

Rhizopus oligosporus used in the present study showed remarkable potential to utilize sunflower meal a by-product of oil industry as cost effective substrate for protease production. In conclusion, the purified acidic protease has molecular weight of 43kDa with an optimum activity at $\text{pH}=4$ and 50°C . However, the suitability of

acidic protease for biotechnological applications on large scale can be investigated through further characterization.

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