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Partial Purification and Characterization of *Arthrobacter globiformis* Protease

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An extracellular protease produced by *Arthrobacter globiformis,* an isolate from the gut of kola nut weevil *Balanogastris kolae* Desbr was partially purified and characterized in this study. The enzyme showed optimum activity at temperature of 60°C and pH 8.0. The molecular weight was estimated to be 35.7 kDa, while the K_m and V_{max} for the hydrolysis of casein were 71.43 mg/min/mL and 8.33mg/ mL respectively. The enzyme retained almost 80% of its relative activity after 20 minutes incubation at 60°C and its activity was not particularly influenced by majority of the cations tested. However, it was inhibited by Hg^{2+} and Fe^{3+}.

Key words: Protease, *Arthrobacter,* Casein, Cations, Weevil.

Protease constitute one of the most important groups of industrial enzymes that are now used in a wide range of industrial enzymes such as in detergent, food, pharmaceutical, leather and silk industries (Adinarayana *et al.*, 2003). Proteolytic enzymes are ubiquitous in occurrence being found in all living organisms. Bacterial proteases are the most significant compared with plant, animal and fungal proteases. They are preferred because they posses almost all characteristics desired for their biotechnological applications (Rao *et al.*, 1998; Alvarez-Sanchez *et al.*, 2000). Protein degrading enzymes constitute about 60% of all enzyme sales (Beg *et al.*, 2003). Enzymes are vulnerable to various environmental factors. Their activity may be significantly diminished or destroyed by a variety of physical or chemical agents resulting in a loss of the functions performed by the enzymes (Pelczar *et al.*, 1986).

Proteolytic enzymes have many physiological functions, ranging from generalized protein digestion to the more specific regulated processes (Mussarat *et al.*, 2000). The success of microbial protease in food and other biotechnological systems could be attributed to the broad biochemical diversity of the microorganisms, genetic manipulation of the organism and the improvement of the techniques in the enzymes production, purification and characterization. Enzymes activity rate are often heavily influenced principally by conditions such as substrate, activators, enzyme concentration, pH redox potential and temperature (Gupta *et al.*, 2002).

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This paper reports on partially purified and characterized protease of produced by *Arthrobacter globiformis*.

MATERIAL AND METHODS

Microorganism and enzyme production: The bacterial strain used in this study was previously isolated from the gut of kola nut weevil. The enzyme was produced in Erlenmeyer flasks containing 500ml of protease production medium containing (g/l): K_2HPO_4, 1.5; KH_2PO_4, 0.5; MgSO4, 0.05; NaCl, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2.2H_2O$, 0.02; $FeSO_4.7H_2O$ 0.02; yeast extract, 0.5; sucrose, 0.5 and 1% casein. The inocula for the experiments were prepared by growing the organism in nutrient broth (NB, Oxoid) at 35°C for 18hrs on a rotary shaker (Gallenkamp). Sterilized medium (500ml) in 1000ml conical flasks was inoculated with 10ml of inocula (1.0×10^3 cells/ml). The flask was incubated at 35°C on a rotary shaker (120r.p.m) for 48hrs and then centrifuged at 5000 rpm for 20mins in cold to remove bacterial cells. The supernatant obtained was used as the crude extract for further studies.

Protease assay

The protease activity was determined in a reaction mixture consisting of 1 mL of substrate solution (1% casein in Tris-HCl buffer, pH 8) and 1 mL of the enzyme solution. The reaction mixture was incubated for 60 minutes at 60°C. The proteins were precipitated by adding 3mL of 0.5% TCA and free amino acids released by protease from casein hydrolysis were estimated by Lowry method. The protease activity was defined as mol of tyrosine released per minute per ml of the enzyme.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at an absorbance of 280nm.

Partial purification of crude protease

The crude protease was fractionated by precipitation with ammonium sulphate (70%) and the precipitate was centrifuged at 10,000 r.p.m for 10 min. The precipitate was re dissolved in Tris-HCl buffer (pH7.8) and dialyzed against several volumes of the same buffer for 24 h at 4°C using acetylated cellophane tubing prepared from Visking dialysis tube (Gallenkamp) as described by Whitaker *et al.* (1963).

Gel filtration chromatography (using Sephadex G-150)

Sephadex G-150 (Sigma, Aldrich) was packed into a column (1.5×75cm) and equilibrated with Tris-HCl buffer (pH 7.8).The column was eluted with the same buffer at a flow rate of 20ml/hour. A fraction of 2.0ml were collected at interval of 30minutes and the absorbance at 280nm was read using spectrophotometer (Jenway, 6305). For determination of molecular weight by gel filtration the standards used were: gamma globulin, 15kDa; alpha chymotrypsinogen, 25.7kDa; ovalbumin, 45kDa; bovine serum albumin, 66kDa and creatine phosphokinase, 81kDa (Sigma, UK).

Characterization of Partially purified enzyme

Effect of temperature on protease activity and stability

Protease activity was assayed by incubating the enzyme reaction mixture at different temperatures, 20°C to 80°C for 1h. The thermal stability at 50°C to 80°C was also determined. Samples were taken at 5minutes intervals and analyzed for protease activity.

Effect of pH on protease activity

Substrates (1% casein) having pH ranging from 5.0 to 8.0 were prepared using 0.05M of different buffer system (Glycine-HCl, pH 3; acetate buffer, pH 4 and 5; phosphate buffer pH 6 and 7; Tris- HCl, pH 8). Enzyme activity was determined at 40°C.

Effect of substrate concentration on protease activity

The effect of substrate concentration [S] on the rate of enzyme action was studied using [S] values of 2.0 mg/ml to 10.0 mg/ml. The Lineweaver-Burke plot was made. Both the V_{max} and K_m of the enzyme were calculated.

Effect of heavy metals on enzyme activity

A stock solution of 0.01M of $HgCl_2$ and EDTA were prepared. Two milliliter of each salt solution was mixed with 2ml of substrate solution. The substrate/ chemical mixture was incubated at room temperature for 5mins before it was used in enzyme assay.

Effect of salts/cations

The effects of various metal Na^+, K^+, Ca^{2+}, Cu^{2+}, Mg^{2+}, and Fe^{2+} (5mM) were investigated by adding them to reaction mixture. Relative protease activities were determined.

RESULTS

The results obtained in this work revealed the ability of the of the *Arthrobacter globiformis* to produce extracellular protease. Fractionation of the enzyme concentrate on Sephadex G-150 produced a double protein peaks with a single peak demonstrating protease activity (Fig. 1). These purification procedures yielded a partially purified protease with specific activity of 33.03 U mg^{-1}

Table 1. Purification of extracellular protease of *A. globiformis*

Fraction	Vol. (ml)	Protein content ($mgmL^{-1}$)	Protease activity (U)	Specific activity(U mg^{-1} of protein)	Yield (%)	Purification fold
Crude enzyme	50	916.5	8600	9.38	100	1.00
$(NH_4)_2SO_4$ precipitation	20	209	3578	17.12	22.8	1.82
Gel Filtration	55	253.2	8364	33.03	27.6	3.52

Table 2. Effect of Salts on the activity of *A. globiformis* protease

Salt	% Relative activity
Control	100
NaCl	97
KCl	82
$CaCl_2$	98
$MgSO_4$	67
$CuSO_4$	48
$FeCl_2$	25
$HgCl_2$	16
EDTA	85

proteins and a purification of approximately five fold with 27.6% yield of proteins (Table 1). The molecular weight of the alpha amylase produced was estimated to be 35.7kDa. The purified enzyme exhibited maximum activity at 60°C (Fig. 2) and pH 8.0 (Fig.3). The protease was almost 70% stable at 60°C even after 120 minutes of incubation (Fig.4). A Lineweaver-Burke plot of the purified protease activity of *A. globiformis* (Fig.5) indicates that this enzyme has apparent K_m of 71.43 mg $ml^{-1}$and V_{max} of 8.33mg/min/ml for the hydrolysis of casein. Cations had no stimulatory effects on the protease, however Hg^{2+} and Fe^{2+}strongly inhibited the activity (Table 2).

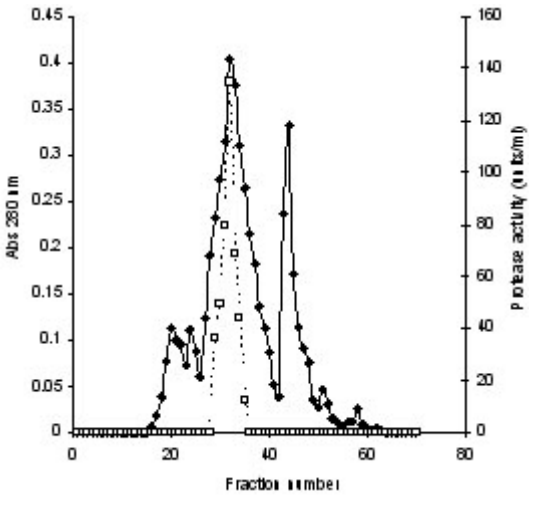

Fig. 1. Elution profile of protease produced by *Athrobacter sp*. grown in production media using sephadex G-150 (1.5 x 75) column equilibrated with 0.1M Tris-HCl buffer, pH 7.8. Flow rate at 20 ml/hr

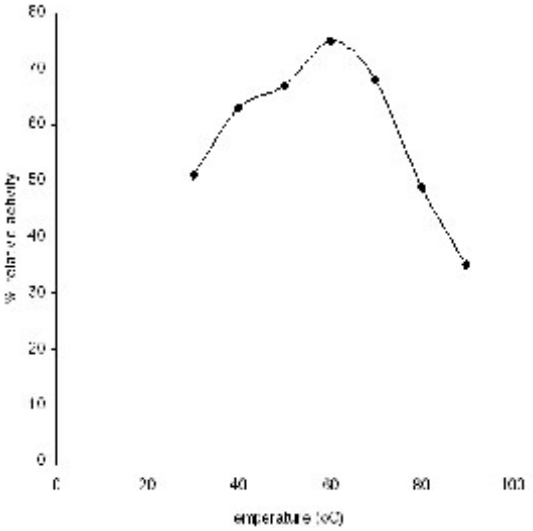

Fig. 2. Effect of temperature on protease activity of *A. globiformis*

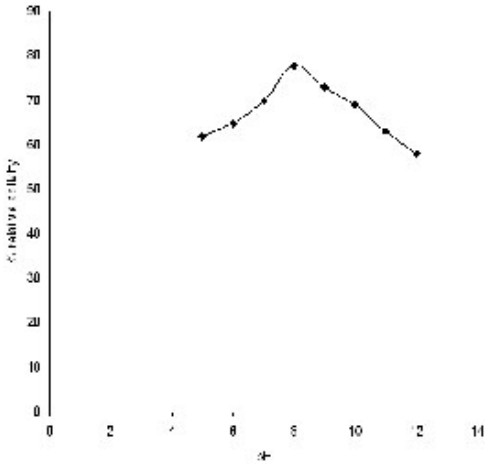

Fig. 3. Effect of pH on protease activity of *A. globiformis*

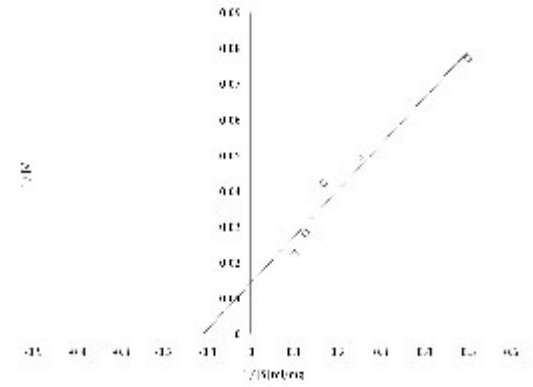

Fig. 5. Line weaver burke plot of protease produced by *A. globiformis*

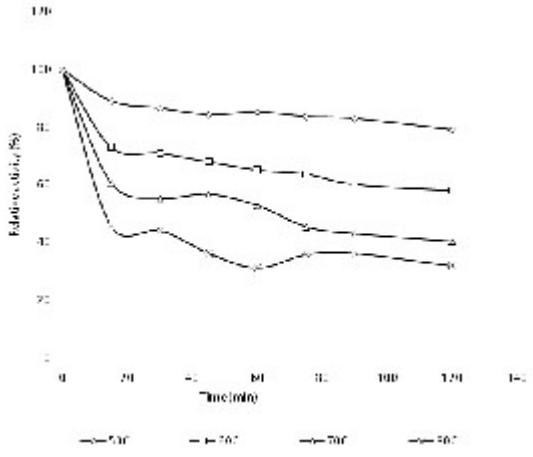

Fig. 4. Thermal stability of α-amylase produced by *Bacillus spp* in a growth medium

DISCUSSION

The molecular weight of *Arthrobacter globiformis* protease in this study was 35.7kDa. This value falls within the range of 18 and 35kDa reported for serine protease (Rao *et al.*, 1998). The protease produced by *A. globiformis* in this study had its optimum activity at pH 8.0. This is within the range for alkaline protease. The optimum pH of purified extracellular protease produced by *Alkaligens faecalis* was 9.0 (Berla and Suseela, 2002). *Ho*wever, Mckevitt *et al.* (1989) reported a lower pH of 6.0 for the protease of *Pseudomonas cepacia* proteinase PSCP. The protease was partially stable at high temperature having an optimum temperature for the protease activity at 60°C, while it retained 50% of its proteolytic activity when heated for 60 minutes at 70°C. Adinarayana *et al.* (2003) also reported an optimum temperature of 60°C for alkaline protease of *Bacillus stearothermophilus* AP-419. Thermal denaturation of enzyme at temperature higher than 70°C has been reported (Aderibigbe, 1998).

In this study, strong inhibition or stimulation of protease activity by metal ions was not observed. The observation correlated with the report of Kim *et al.* (2001) that alkaline protease are generally neither inhibited by metal chelating agents nor activated by metal ions or reducing agents. However some other authors have reported increase in protease activity by divalent ions like Ca^{2+} particularly for metalloprotease (Jaswal and Kocher, 2007). Hg^{2+} and Fe^{2+}strongly inhibited the activity of the protease of *A. globiformis*. The inactivation of enzymes via heavy metal poisoning has been well documented (Dixon and Webb, 1971). They are known to react with protein sulphydryl groups, thus converting them to mercaptides.

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