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Partial purification and some properties of *Vibrio haemolyticus protease*

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Abstract

An extracellular protease of *Vibro haemolyticus* isolated from the gut of kolanut weevil *Balanogastris kolae* Desbr was partially purified and characterized in this study. The enzyme was purified in a two-step procedure involving ammonium sulphate precipitation and Sephadex G-150 gel filtration chromatography. The purified protease had an apparent molecular weight of 22.3kDa. The protease was found to have optimum activities at temperature of 50^{o}C and pH of 8. The maximum velocity V_{max} and K_m of the protease produced during the hydrolysis of casein were 66.66mg/min/mL and 3.33mg/mL. The enzyme was almost 100% stable at 60^{o}C even after 120 minutes of incubation. It was strongly activated by K^{+}, while the enzyme activity was strongly inhibited by Fe^{3+} and Hg^{2+}. .

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Introduction

Protease represents the class of enzymes that occupy a position with respect to their physiological roles as well as their commercial applications (Mussarat *et al.*, 2000). A variety of microorganism such as bacteria, yeast, moulds and Actinomycetes are known to produce proteases (Madan *et al.*, 2002). Plant and animals are also sources of proteases, however microbial proteases are among the most important hydrolytic enzyme and they have been studied extensively (Gupta *et al.*, 2002). Extracellular protease are of commercial value and has found multiple application in various industrial sectors such as pharmaceutical, detergent, laundry and tanning (Gupta *et al.*, 2002; Norris, 2003). Proteases constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales (Beg *et al.*, 2003). Fujiwura and Yamamoto (2007) had also reported the use of protease in the decomposition of gelatinous coating in x-ray films. Proteases occur naturally in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades. Bacteria also secrete proteases to hydrolyze the peptide bonds in protein and therefore break the proteins down into constituent monomers (Prescott *et al.*, 1999).

A myriad of organisms from different exotic environments have been exploited for protease production, particularly alkaline protease. Selection of the right organisms plays a key role in high yield of desirable enzymes. Isolation of new promising strains from different environments is a continuous process. In this present study, the extracellular protease produced by *Vibrio haemolyticus*, an isolate from the gut of kolanut weevil, *Balanogastris kolae* Desbr was partially purified and characterized.

Materials and methods

Organisms and culture conditions

The *Vibrio haemolyticus*, an isolate from the gut of kolanut weevil, *Balanogastris kolae*, was used in the production of protease. The organism was grown in a basal medium containing (g/l): K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4$, 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 r.p.m 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 280 nm.

Purification and characterization of alpha amylase

All chromatography procedures were carried out at 4°C except where stated otherwise.

Ammonium sulphate precipitation

Fifty milliliters (50 mL) of crude enzyme was precipitated (fractional) with $(NH_4)_2SO_4$ (Analytical grade, B.D.H) at 70% (w/v) saturation. 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).

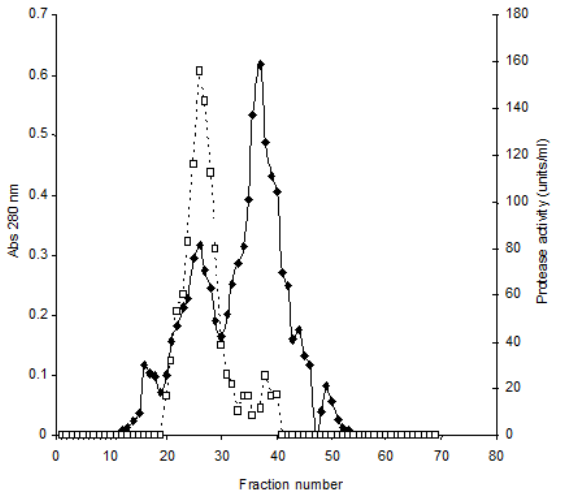

Fig. 1. Elution profile of protease produced by *Vibrio haemolyticus* on Sephadex G – 150 (1.5 x 75) column equilibrated with 0.1M Tris – HCl buffer, pH 7.8. Flow rate at 20 ml/hr. Abs 280 nm (♦♦♦♦♦♦♦♦♦♦), Protease activity (µmol/min/ml)

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).

Table 1. Purification of extracellular protease of *V. haemolyticus*.

Fraction	Vol. (ml)	Protein content ($mgmL^{-1}$)	Protease activity (U)	Specific activity(U $mg^{-1}$of protein)	Yield (%)	Purification fold
Crude enzyme	50	1244.5±0.2	5133±0.5	4.12±0.08	100.	1.00
$(NH_4)_2SO_4$ precipitation	20	406.8±0.3	2840±0.4	6.98±0.02	32	1.69
Gel Filtration	55	520.3±0.5	11077±0.25	21.29±0.01	41	5.16

Values represent the mean of duplicate determinations.

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 was 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.

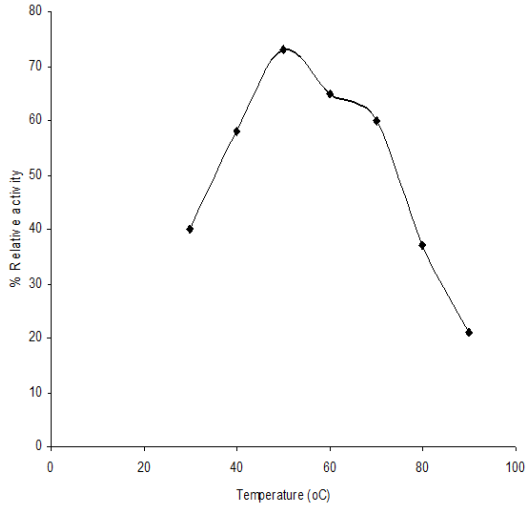

Fig. 2. Effect of temperature on the activity of partially purified protease of *Vibrio haemolyticus*.

Effect of cations

A stock solution of 0.01M of each salt was prepared. The effects of some salts/cations (NaCl, KCl, $CaCl_2$, $CuSO_4$, $MgSO_4$, and $FeCl_2$) on enzyme activity was also determined. The substrate/salts mixture was also incubated before it was used for enzyme assay.

Results

Fractionation of the enzyme concentrate on Sephadex G-150 produced a double peak (Fig. 1). These purification procedures yielded a partially purified protease with specific activity of 21.28U mg^{-1} proteins and a purification of approximately five fold with 41% yield of proteins (Table 1). The molecular weight of the alpha amylase produced was estimated to be 22.3kDa. The purified enzyme exhibited maximum activity at 45°C (Fig. 2) and pH 7.5 (Fig.3). The protease was almost 100% stable at 60°C even after 120 minutes of incubation (Fig.4). At a temperature of 80°C, the enzyme retained about 40% of its activity after 120 minutes of incubation. A Lineweaver-Burke plot of the purified protease activity of *V. haemolyticus* (Fig.5) indicates that this enzyme has apparent K_m of 66.66 mg $ml^{-1}$and V_{max} of 3.33mg/min/ml for the hydrolysis of casein. The activity of the protease was stimulated by K^+, while EDTA, Ca^{2+} and Mg^{2+} acted as mild inhibitors. Fe^{3+} and Hg^{2+} strongly inhibited the activity (Table 2).

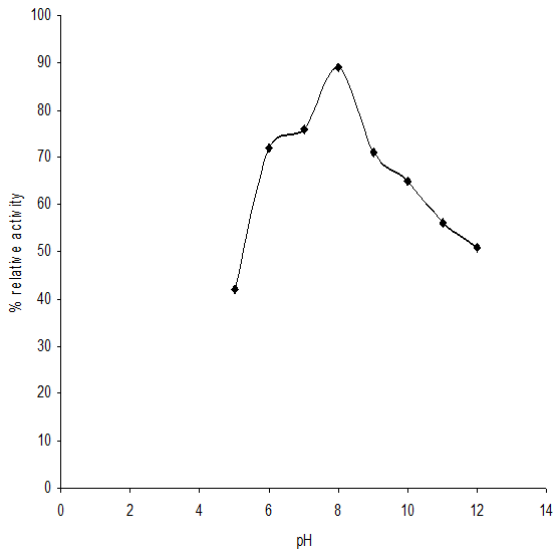

Fig. 3. Effect of pH on activity of partially purified protease of *Vibrio haemolyticus*.

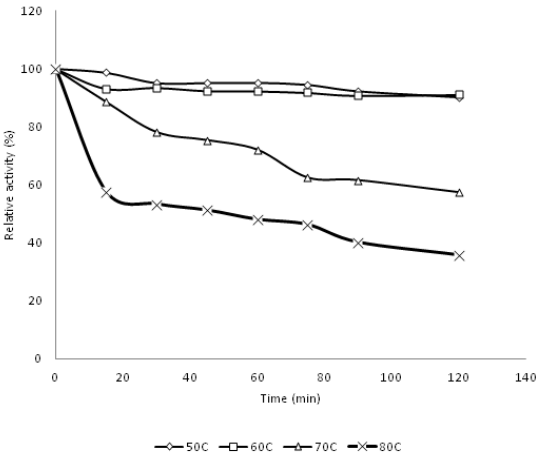

Fig. 4. Thermal stability of protease produced by *Vibrio haemolyticus*.

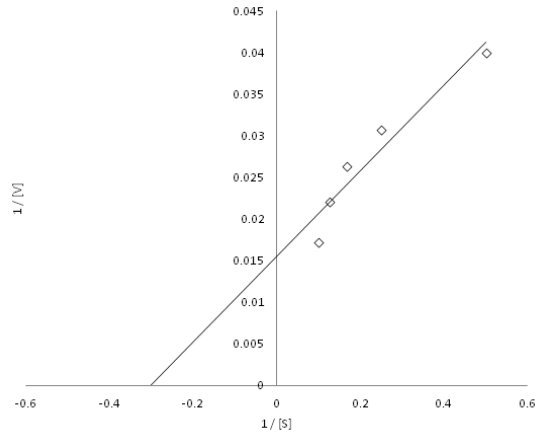

Fig. 5. Lineweaver- Burke plot of protease produced by *Vibro haemolyticus*.

Table 2. Effect of salts on the activity of *Vibrio haemolyticus* protease.

Salt	% Relative activity
Control	100
NaCl	76
KCl	103
$CaCl_2$	91
$MgSO_4$	88
$CuSO_4$	35
FeCl2	56
$HgCl_2$	45
EDTA	68

Discussion

The result of this study showed that *V. haemolyticus* protease was partially stable at high temperature. The optimum temperature for the protease activity was 50°C, while it retained 70% of its proteolytic activity when heated for 60 minutes at 70°C. Lee *et al.* (2002) had also reported similar temperature optimum for *V. parahaemolyticus* protease. Adinarayana *et al.* (2003) however 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).

Various cations and inhibitors tested had different effects on the protease activity of *V. haemolyticus*. In this study, strong inhibition or stimulation of protease activity by metal ions was not observed. It has been reported that alkaline protease are generally neither inhibited by metal chelating agents nor activated by metal ions or reducing agents (Kim *et al.*, 2001).However some other authors have reported increase in protease activity by divalent ions like Ca^{2+} particularly for metalloprotease (Jaswal and Kocher, 2007). Only K^{+} activated the protease activity of *V. haemolyticus*.The molecular weight of *V. haemolyticus protease* in this study was 22.3kDa. This value falls within the range of 18 and 35kDa reported for serine protease (Rao *et al.*, 1998).

The findings in this study suggest that the protease is a suitable candidate for biotechnological applications. The stability of the enzyme to heat may be an indication of its possible application in industrial processes.

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