Extracellular Novel Metalloprotease from *Xenorhabdus indica* and Its Potential as an Insecticidal Agent

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**Introduction**

*Xenorhabdus*, an endosymbiont of *Steinernema*, has a unique life cycle causing pathogenicity in the insect host [1, 3]. The infective juveniles (IJ’s) of Steinernematidae release the bacterium into the nutrient-rich hemolymph within 5 h of invasion [4, 31]. The bacteria proliferate and produce a wide range of toxins, antibiotics, bacteriocins, and hydrolytic exoenzymes, resulting in a bacterial septicemia and death of the host within 24–48 h [12, 18]. The bacteria also provide a suitable nutrient-rich environment for nematode growth and reproduction. The immature nematode develops in the IJ’s that again carry away the bacteria, in search of a new insect host to continue the cycle. A very interesting significant feature of bacterium *Xenorhabdus* is that there are two phenotypic variants, primary (Phase-I) and secondary form (Phase-II). They differ in their antibiotic production profile, outer membrane proteins (fimbriae and flagellae), symbiotic capabilities with nematode partner, and exoenzyme production potential. The complete genomic analysis of *Xenorhabdus* has shown that it has many genes that encode different toxins, proteases, and lipases [9]. The protease enzyme plays
an important role in the pathogenicity of the nematode–bacteria complex. It is a well accepted phenomenon that secreted proteolytic enzymes of *Xenorhabdus* play a significant role in virulence by suppressing the immune response of the insect host and helping in tissue penetration [23]. Unraveling such systems of the pathogen, secreted proteases can provide insight regarding their role in a host’s defense mechanism. Surprisingly, despite the importance of proteases in insect pathogenesis, only a few studies have been undertaken to explore the nature of protease and its production under axenic cultivation based upon their substrate specificity [11].

In this paper, we have for the first time reported the isolation and characterization of an alkaline metalloprotease from different isolates of *Xenorhabdus* species. *X. indica* has been found to produce the maximum of secreted alkaline protease. The MALDI-TOF/TOF analysis of the homogeneously purified protease confirmed its identity as secreted alkaline metalloprotease from *Xenorhabdus*. The bioefficacy of the purified protease was evaluated against *H. armigera* (cotton bollworm).

**Materials and Methods**

**Bacterial Strain and Growth Condition**

The strains of *Xenorhabdus* sp. were isolated from IJ’s of different Steinernema sp. The primary form was differentiated on the basis of characteristic blue colony on NBTA medium (Peptone 5; Beef extract 3; NaCl 4; Bromothymol blue 0.025; Triphenyl-2,3,4-tetrazolium chloride 0.04 in g/l), unlike the secondary form, which had a chocolate brown color.

**Screening of *Xenorhabdus* Isolates for Protease Enzyme**

**Qualitative assay.** Qualitative assay of protease enzyme was carried out on gelatin agar plates (Peptone 5; Beef extract 3; NaCl 4; Gelatin 12 in g/l, pH 7.2) by spot inoculating 2 µl of cell suspension and incubation at 28°C [21]. After 48 h, the plates were flooded with 5 ml of HgCl₂ solution and the zone of hydrolysis observed. On the basis of the qualitative assay, the most promising *Xenorhabdus* strain was selected for further studies.

**Selection of Medium for Protease Enzyme**

For optimum production of protease enzyme, five different media were evaluated: skim milk (1%) in nutrient broth (NB), skim milk (1%) in NB (Half strength), gelatin (1%) in NB, gelatin (1%) in NB (half strength), soya casein digest medium. The media were inoculated with 2% (v/v) cell suspension and incubated at 28°C, 150 rpm for 24 h. Samples were withdrawn at intervals of 3 h upto 24 h for the enzyme assay.

**Enzyme Assay**

The culture suspension was centrifuged at 10,000 rpm for 5 min and culture supernatant was used as the enzyme source. Protease activity was assayed by incubating 250 µl of azocasein (Megazyme, 2% (w/v)) with 150 µl of enzyme solution in a water bath at 30°C for 30 min [28]. After incubation 1.2 ml of 10% trichloroacetic acid was added to stop the reaction and the mixture was allowed to stand for 15 min. Enzyme blanks were prepared by mixing buffer, azocasein, trichloroacetic acid, and enzyme. The content was centrifuged at 10,000 rpm for 5 min to remove any undigested azocasein. The optical density of reaction supernatant was determined by adding 1.4 ml of NaOH (1 N) in supernatant (1.2 ml). One unit of enzyme is defined as the amount of enzyme required to produce an absorbance change of 0.01 in a 1 cm cuvette under the standard assay conditions.

**Purification of Protease Enzyme**

Extracellular protease enzyme was extracted by centrifugation of cell suspension grown in soya casein digest medium at 10,000 rpm after 18 h of incubation. Cell-free supernatant was saturated with ammonium sulfate (80%), and precipitate was collected after centrifugation at 10,000 rpm, dissolved in 0.1 M TrisHCl buffer (pH 7.6), and dialyzed overnight at 4°C against the same buffer. The dialysate was ultrafiltered using a 30 kDa Amicon ultra filtration unit (Millipore) followed by anion-exchange chromatography using a Macro-Prep High Q (Biorad) pre-packed column equilibrated with the same buffer. The protein was eluted with a NaCl gradient (0.5–1.5 M) in the same buffer at a flow rate of 1 ml/min. Fractions having protease activity were pooled and concentrated using a 3 kDa Amicon ultra filtration unit. The purified fraction was stored at −20°C, and its purity verified using SDS-PAGE and zymography [19, 30].

**Characterization of Protease Enzyme**

**Determination of optimum pH and temperature.** The optimal pH of partially purified protease enzyme was determined in 0.1 M sodium phosphate buffer (pH 6.2–7.4), 0.1 M TrisHCl buffer (pH 7.8–8.6), and 0.1 M Glycine NaOH buffer (pH 9.0–9.8). The optimal temperature was investigated by exposure of enzyme to temperatures in the range of 26–42°C. After exposing the enzyme to different pH and temperature values for 30 min, the protease activity was estimated as per the standard protocol described earlier.

**Effect of metal ions and inhibitors.** The effect of metal ions were determined in the presence of different metals such as Mn²⁺, Ca²⁺, Zn²⁺, Co²⁺, Cu²⁺, Fe³⁺, and Mg²⁺ at a concentration range 5–25 mM. The effect of inhibitors was studied using EDTA, PMSF, and 1,10-phenanthroline (10–25 mM) on the protease activity.

**Enzyme kinetics.** Kinetic constants such as *Vₘₐₓ* and *Kₘ* were identified under steady-state conditions using various concentrations of azocasein (5–30 mg/ml) as substrate [29].

**Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF/TOF)**

Purified protein sample was digested with sequencing grade trypsin according to the manufacturer protocol [20]. In brief,