

Characterization of an Alkaline Metalloprotease Secreted by the Entomopathogenic Bacterium *Photorhabdus* Sp. Strain EK1

¹Magda A.M. Soroor, ²Hoda H. El Hendawy, Abd-Elhady M. Ghazy, ²Nermeen A. El Semary,
³Kamal M.A. Khalil, Ahmed M. Abd El Aziz

¹Molecular Biology Department, ³Genetics and Cytology Department,
National Research Centre, Cairo, Egypt.

²Botany and Microbiology Department, Faculty of Science, Helwan University, Cairo, Egypt.

Abstract: A purified alkaline protease from entomopathogenic bacterium *Photorhabdus* sp. Strain EK1 (*PhPrtpI*), a bacterium pathogenic for insects and is symbiotic with an Egyptian nematode of the genus *Heterorhabditis*, has been characterized. The enzyme has a molecular weight of 25 and 23kDa for native and denatured enzyme, respectively; suggesting that the enzyme is monomeric. *PhPrtpI* had an optimum temperature at 40°C for both enzyme activity and stability. The activation energy for the hydrolysis of azocasein was estimated to be 5.2kcal/mol. The enzyme activity was enhanced by Ca²⁺, while Zn²⁺ has no stimulatory or inhibitory effects. Most of the enzyme activity was inhibited by EDTA with Ki 9.4mM and partially inhibited by 1,10-phenanthroline, but not affected by cysteine, serine and aspartyl protease inhibitors. *PhPrtpI* had an optimum pH of 8.5 and classified as alkaline metalloprotease and it is Ca²⁺ dependent. Its substrate specificity strengthens the possibility that *PhPrtpI* involved in degradation of insect tissues for providing nutrients to the associated nematode, which is unable to develop and reproduce inside the infected insect without a previous bioconversion of the insect cadavers by the symbiotic bacterium. Its biochemical characteristics were compared with those previously reported for different species of animal pathogenic bacteria.

Key words: alkaline, characterization, entomopathogenic nematode, *Heterorhabditis*, metallo, *Photorhabdus*, protease, symbiotic bacterium.

INTRODUCTION

The bacteria of the genus *Photorhabdus* are gram-negative organisms of the family *Enterobacteriaceae* that live as symbionts in the intestine of the entomopathogenic nematodes (EPNs) of the family *Heterorhabditidae*^[19,26]. The two partners are voracious pathogens to a wide variety of insect pests and currently used as biocontrol agents. The nematodes live in the soil where they seek out and enter insect hosts. The bacteria once expelled from the nematode enter the hemocoel of the insect, actively multiply avoiding the host defenses and causing an acute disease conditions that is followed by insect death within 48 hours^[33].

Within the insect carcasses, the bacteria create the nutritional conditions and protective environmental for the development of its nematode symbionts^[14,23,9,26]. They are secrete an array of hydrolytic exoenzymes

including lipases, phospholipases, chitinases and proteases that are responsible for the death and bioconversion of the insect cadavers into a nutrient soap that is ideal for nematode growth and reproduction. The final stage of EPN development is the re-association of the bacteria and nematodes to form non-feeding infective juveniles (IJ) which emerge from the insect cadavers to find a new host^[20,9].

Bacterial proteases are mainly involved in providing peptide nutrients for the microorganisms. However, the production of bacterial proteases might contribute to the pathogenesis of infections and therefore they could be considered as one of the extracellular virulence factors. Alkaline metalloproteases have been purified and characterized from different species of *Photorhabdus* symbiont of the EPNs *Heterorhabditis* species^[6,52,7,9,26] and *Xenorhabdus* symbiont of the *Steinernema* species^[30,12,38].

Correspondence Author: Magda A.M. Soroor, Molecular Biology Department, National Research Centre, Dokki 12622, Giza, Egypt.
Tel.: (202) 3335980-Extension: 1733 Fax: (202) 3370931
Email: magdasoroor@yahoo.com, magdasoroor@email.com

The entomopathogenic bacterium *Photorhabdus* sp. strain EK1 has been isolated from the Egyptian heterorhabditid nematode isolate, *Heterorhabditis bacteriophora* EK1. Two alkaline proteases PI and PIII have been secreted by such bacterium at the stationary growth phase at 48-h postinoculation. These two isoenzymes were purified and one of them (PIII) has been characterized as an alkaline metalloprotease^[38]. The present study was designed to characterize the second isoenzyme of *Photorhabdus* alkaline protease PI in order to compare its physical, chemical and functional properties with those previously reported from different species of pathogenic bacteria as well as to clarify the role/s of such enzyme in virulence and survival of heterorhabditid nematodes inside an insect cadaver.

MATERIALS AND METHODS

Nematode Source and Isolation of Bacteria: The IJs of the EPN *H. bacteriophora* strain EK1 were isolated from El Kaliobyia, Egypt and maintained in the late instar larvae of the greater wax moth, *Galleria mellonella*, at 27°C±1°C as described by Woodring and Kaya^[54]. The IJs were collected within 2-4 days of their emergence from the insect cadavers, washed three times by sedimentation in tap water and finally in distilled water.

Bacterial Isolation: The bacterium *Photorhabdus* sp. strain EK1, is a symbiont of the heterorhabditid nematode *H. bacteriophora* EK1, was isolated according to Caldas *et al.*^[10] and Cabral *et al.*^[9] The IJs were surface sterilized for 2 min in 0.1% sodium hypochlorite, washed in sterile distilled water several times, transferred to a petri dish containing 5 ml of Luria-Bertani (LB) medium plates (1% pepton, 0.5 % yeast extract, 0.5% NaCl) and cut into several pieces by blades. The plates were incubated at 28°C for 24 h and single suitable colony was spread on medium plates of nutrient Bromothymol agar (NBTA) (3.3% nutrient agar, 0.0025 % bromothymol blue and 0.004% 2,3,5-triphenyltetrazolium chloride). The plates were incubated for 48 h at 28°C. The isolated bacterium was maintained on NBTA plates at 10°C and is subcultured weekly. The isolated bacterium was cultured in liquid culture medium (LCM) (10% nutrient broth, 40% trypton soya extract, 5% pepton from casein, 5% yeast, 5% NaCl, 0.035% KCl and 0.021% CaCl₂. 2H₂O) and maintained in -80°C. The bacteria colonies used in this study were phase I variant because they showed a greenish blue color on NBTA medium, absorption of dyes and bioluminescence according to Akhurst^[4].

Enzyme Assay: The proteolytic activity was determined as described by Tomarelli *et al.*^[50] and

modified by Cabral *et al.*^[9] Azocasein was used at a final concentration of 0.2 % in a 1.0 ml assay reaction mixture containing 100 mM Tris-HCl buffer, pH 8.5 and appropriate amount of enzyme. The reaction mixtures were incubated for 1 h at 37°C and terminated by adding 0.5 ml of 15% (w/v) trichloroacetic acid. They were incubated for 15 min at 4°C, followed in turn by centrifugation at 5,000Xg for 3 min at room temperature and the absorbance was recorded at 366 nm. One unit of proteolytic activity was defined as µg azocasein hydrolyzed per hour under standard assay conditions according to Brock *et al.*^[8].

Buffers: Buffers were prepared according to Gomori^[23] and the final pH was confirmed with a pH meter.

Molecular Weight Determination: Molecular weight was determined by gel filtration technique using Sephacryl S-200 column (95x1.6 cm i.d.). The column was calibrated with cytochrome C (12,4 kDa), carbonic anhydrase (30 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa). Dextran blue (2000 000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis^[32]. SDS-denatured phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14,4 kDa) were used as marker proteins for the calibration curve.

Effect of Ph on Enzyme Activity: The effect of pH on the activity of *Photorhabdus* sp. EK1 protease PI (*PhPr*PI) was examined over the range pH 6-10 using 100 mM of each: sodium phosphate buffer for pHs 6.0 to 7.5, Tris-HCl buffer for pHs 7.5 to 9 and glycine-NaOH for pHs 9.0 to 10.5.

Effect of Temperature on Enzyme Activity and Stability: To examine the effect of temperature on the activity of *PhPr*PI, the complete enzyme reaction mixtures were incubated at different temperatures ranging from 25 to 80°C. For estimating the effect of temperature on the enzyme stability, the enzyme was preincubated for 30 min at different temperatures ranging from 25 to 80°C, followed by cooling and measuring the remaining activity as described previously in Materials and Methods Section.

Inhibition Studies: To determine the class, to which the *PhPr*PI belongs, azocaseinolytic activity was measured in presence of several inhibitors and activators. The enzyme was incubated with phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), ethylenediaminetetracetic acid (EDTA), leupeptin, pepstatin A, N-ethylmaleimide (NEM), 1,10-

phenanthroline, dithiothreitol (DTT), β -mercaptoethanol and cysteine individually for 30 min at room temperature and followed by measuring the remaining activity.

Substrate Specificity: The substrate specificity of *PhPrtPI* was performed as previously described using casein, hemoglobin, albumin, fibrin, collagen and gelatin as substrates. After precipitation of the undigested proteins and centrifugation, the absorbance of the supernatants were recorded at 280 nm according to Abu-Hatab *et al.*^[2].

RESULTS AND DISCUSSION

After the release of entomopathogenic bacteria, *Photorhabdus* and *Xenorhabdus* spp., into the insect hemocoel by the EPNs, the bacteria secrete a variety of toxins and enzymes including lipases, phospholipases, chitinases and proteases to enable the bacteria and nematode to colonize and reproduce inside an insect host^[24,34,47,26,38]. Proteases represent the important part of the extracellular enzymes. Previous studies on the extracellular proteases have been equivocal as to their role in insect toxicity. While Bowen *et al.*^[6] suggested that extracellular proteases secreted by the bacterium *P. luminescens* are participated in the activation of the toxic complex, Morgan *et al.*^[40] and Caldas *et al.*^[10] suggested that they are active against insect immune factors and have a specific role in attacking the antibacterial defense system of the insect. However, others have found no correlation between extracellular proteases and insect toxicity^[6]. Recently, Mohamed and Hussein^[38] suggested that an alkaline protease produced by the bacterium *X. nematophila* BA2 participates in the bioconversion of the insect cadavers into a nutrient soap for providing nutrients to the associated nematode.

In the present study, we are interested to characterize the second isoenzyme of an alkaline protease PI that is previously purified from the culture medium of 48h postinoculation of the bacterium *Photorhabdus* sp. strain EK1 to address its significant role/s in survival of the EPNs inside an insect host.

Characterization of *Photorhabdus* Alkaline Protease Pi:

The Molecular Weight of Alkaline Protease Pi: The molecular weight of the native *PhPrtPI* enzyme was estimated from the calibration curve (Fig. 1) found to be 25 kDa by gel filtration on Sephacryl S-200 column (Fig. 2). This value was confirmed by SDS-PAGE, where the enzyme migrated as single protein band and was found to be 23 kDa (Fig. 3) indicating that the purified enzyme is a monomeric. This value is congruent to that recorded for alkaline proteases from

Pseudomonas sp. strain DY-A (25kDa)^[55]. The molecular weight of *PhPrtPI* is comparable equal to that estimated for different species of *Bacillus* (29.0 kDa)^[31,25]. However, it is 1.6- fold higher than calculated for *B. subtilis* BE-11^[3]. On the contrary, *PhPrtPI* has a molecular weight which represents 1.7-2.5 and 1.4-3.1 fold lower than that estimated for different species of *Xenorhabdus*^[30,10] and *Photorhabdus*^[6,35,9].

Effect of Temperature on Enzyme Stability: The stability of *PhPrtPI* at temperatures above 45 °C was rather low. The enzyme lost 45 and 85% of its activity upon incubation for 30 min at 50 and 60 °C respectively (Fig. 4). Similar observations have been recorded for *Pseudomonas aeruginosa* metalloprotease^[21] where only 30 min of incubation at 50 °C caused an inactivation of 50% of the enzyme activity. The thermolability of *PhPrtPI* herein is also agrees with that recorded for alkaline protease from *Bacillus pumilus* where 53 and 85% loss in the enzymatic activity was observed at 50 and 60 °C respectively^[27]. In contrast, 93 and 65% of metalloproteases, PrtA and PrtS activities of *Photorhabdus* sp. strain Az29 were retained upon incubation for 30 min at 60 °C respectively^[9]. Alkaline proteases produced by *P. luminescens* K122 and *P. temperate* were also retained 63% of their enzymatic activities upon incubation at such conditions^[7].

Effect of Temperature on Enzyme Activity: The enzymatic activity of *PhPrtPI* was increased by increasing the temperature and the optimal temperature for activity was observed at 40 °C (Fig. 4). From 45°C onward, the enzymatic activity declined rapidly where more than 80% of the proteolytic activity was lost at 70°C. The optimal temperature for activity of *PhPrtPI* was comparable to that recorded for proteases purified from psychrophilic bacterium *Pseudomonas* strain DY-A^[55], fish pathogen *Y. ruckeri*^[44] and from *P. aeruginosa*^[21]. The optimal temperature for activity of *PhPrtPI* is higher by 1.7- fold than that estimated for *X. nematophila* protease II^[10] and *X. nematophila* BA2^[38]. On the contrary, it is lower by 1.25- fold than that recorded for *Photorhabdus* strain Az29 metalloprotease PrtA (50°C)^[9].

Using the temperature range from 25 to 40°C, the activation energy (AE) for *PhPrtPI* is 5.2 kcal/mol (Fig.5). This value is in consistent with that recorded for fish pathogen *F. psychrophilum* protease (5.6 kcal/mol)^[45]. In contrast, it is lower by 3.1, 3.0 and 4.6-fold than that estimated for purified proteases from *X. nematophila* BA2 (16.1 kcal/mol)^[38] *Y. ruckeri* (15.5 kcal/mol)^[44] and from *Bacillus clausii* (24.1 kcal/mol)^[29] respectively.

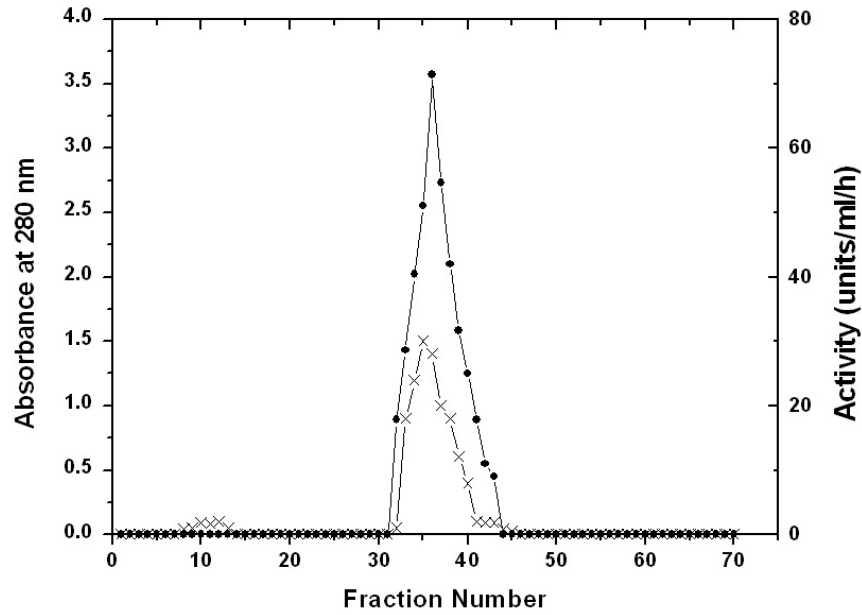


Fig. 1: Gel filtration of purified *PhPr*PI on Sephacryl S-200 column (95 x 1.6 cm i.d.) that is previously equilibrated with 20 mM Tris-HCl buffer, pH 8.5 at a flow rate of 20 ml/h and 3 ml fractions. Absorbance at 280 nm (x---x) and alkaline protease PI activity (●---●).

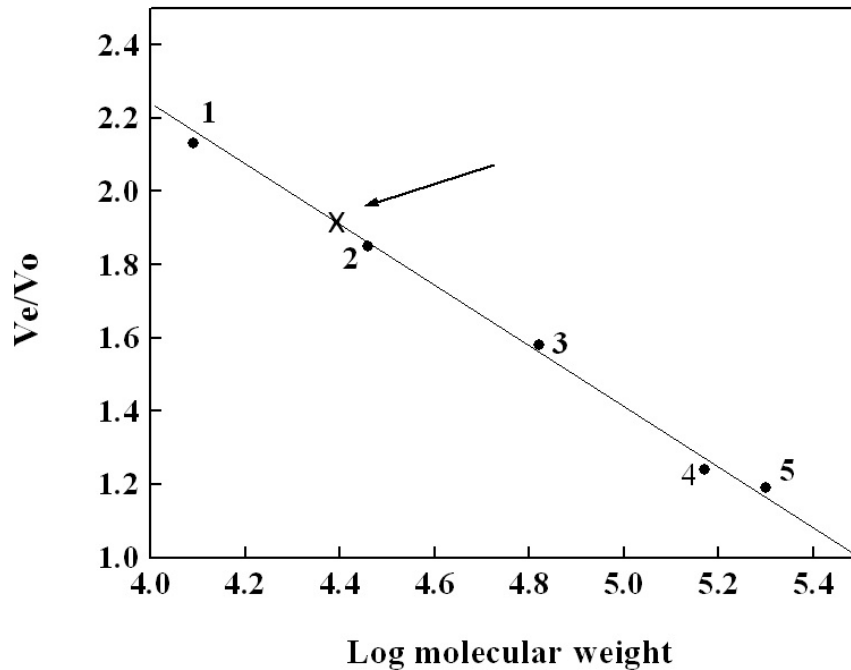


Fig. 2: Calibration curve for the molecular weight estimation by gel filtration on the Sephacryl S-200 column (90 X 1.6 cm i.d.). 1- Cytochrome C (12,4 kDa), 2- Carbonic anhydrase (30 kDa), 3- Bovine albumin (67 kDa), 4- Alcohol dehydrogenase (150 kDa), 5- β -Amylase (200 kDa). The void volume (V_o) was determined with dextran blue (2,000 kDa).

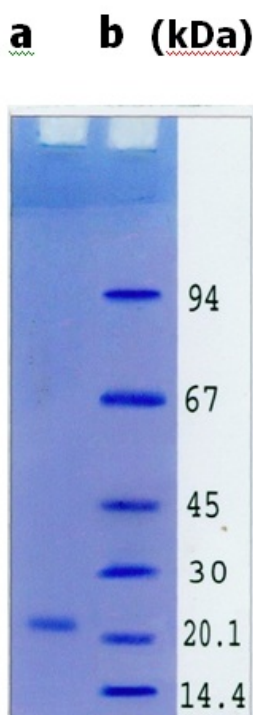


Fig. 3: SDS-PAGE for molecular weight determination of *PhPrtPI*. (a) PI isoenzyme. (b) molecular weight marker.

Effect of Metal Ions: The effect of metal ions on the enzymatic activity of *PhPrtPI* revealed that Fe^{3+} , Ba^{2+} , Mg^{2+} and Ca^{2+} have stimulatory effect and ranged from 33- to 83% (Table 1). This result is in agreement with microbial alkaline proteases purified from *Pseudomonas aeruginosa* strain K^[1] fish pathogens *F. psychrophilum*^[45] and *Y. ruckeri*^[44] and from different species of *Bacillus*^[31,32,27,25] that are reported to be activated by Ca^{2+} . The enzyme enhancement by Ca^{2+} has also been observed for alkaline proteases from entomopathogenic bacteria including *P. luminescens* strain W14 and K122^[7] and *X. nematophilus*^[12]. While Zn^{2+} has no stimulatory or inhibitory effects on *PhPrtPI*. Also Zn^{2+} has no significant effect on alkaline proteases from *P. luminescens* strain W14 and K122 (Bowen *et al.*, 2003), *F. psychrophilum*^[45] and different species of *Bacillus*^[3,27,25].

On the contrary, Cu^{2+} , Ni^{2+} , Hg^{2+} and Co^{2+} ions exhibited inhibitory effects on *PhPrtPI* and ranged from 48- to 84%. While Cu^{2+} and Ni^{2+} strongly inhibited alkaline proteases from *Staphylococcus aureus*^[48], *P. aeruginosa* PseA^[25] and *A. faecalis*^[49], they are moderately inhibited *PhPrtPI*. The enzyme activity of *PhPrtPI* was reduced to 33% by Hg^{2+} , the enzyme activities of *B. pumilus* strain MK6-5^[31] and *A. faecalis*^[49] were abolished by such ion (Table 1).

Table 1: Effect of metal cations on the activity of *PhPrtPI*.

Cations	Relative activity (%)
None	100%
Ba^{2+}	133%
Ca^{2+}	183%
Co^{2+}	16%
Cu^{2+}	52%
Fe^{3+}	126%
Hg^{2+}	33%
Mg^{2+}	137%
Mn^{2+}	10%
Ni^{2+}	52%
Zn^{2+}	100%

Enzyme was pre-incubated for 30 min at room temperature with 10 mM of listed cations as final concentration prior to substrate addition. Activity without added metal cations was taken as 100 %. Each value represents the average of two experiments.

Effect of Inhibitors: The sensitivity of *PhPrtPI* towards different classes of inhibitors and activators was carried out to determine the family at which the enzyme belongs. The results revealed that none of the serine and cysteine protease inhibitors inhibited *PhPrtPI* nor did the aspartyl protease inhibitor (Table 2). However, the enzyme was inhibited strongly (80%) by the metal chelators EDTA (a general metalloprotease inhibitor) and partially (25%) by 1,10-phenanthroline (a specific zinc metalloprotease inhibitor) respectively. These results suggested that the *PhPrtPI* belongs to a metalloprotease family.

The inhibitory effect of EDTA on *PhPrtPI* was increased by increasing EDTA concentration and time of incubation (Fig.7 a, b). The percent inhibition 52.9 and 80 was recorded upon incubation of the enzyme with 10 and 20 mM EDTA respectively. Similar behavior has been observed for *P. luminescens* strain K122 (Bowen *et al.*, 2003) and *P. luminescens* ssp. *laumondii* strain Brecon^[36] alkaline metalloproteases where the inhibitory effect was increased by increasing EDTA concentration. The inhibition constant (K_i) of EDTA as a competitor for *PhPrtPI* was calculated to be 9.4 mM (Fig. 6).

For identification of the divalent cation that is required for the enzyme activity of *PhPrtPI*; Ca^{2+} , which is known to be essential for microbial metalloproteases, was added to the reaction mixture containing EDTA-inhibited enzyme complex. Most of the enzyme activity was restored (93%) by 20 mM Ca^{2+} (Table2). These results suggested that *PhPrtPI* can be classified as a metalloprotease dependent on Ca^{2+} for its activity. The dependence of alkaline metalloproteases on Ca^{2+} has been reported for *Y. ruckeri*^[44], *F. psychrophilum*^[45] and *P. aeruginosa*^[21].

No stimulatory effects were observed upon incubation of *PhPrtPI* with low and high (0.1 and 10 mM) concentrations of dithiothreitol (DTT), cysteine and β - mercaptoethanol (Table 2). On the contrary, an inhibitory effect ranged from 52.9 to 64.7% was recorded and such inhibitory effects increased by

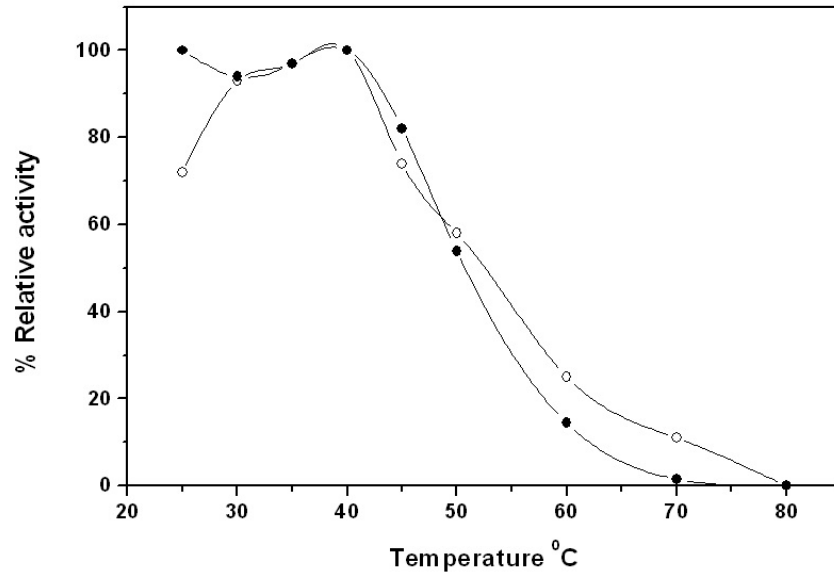


Fig. 4: The effect of temperature on the stability (●-●) and activity (o-o) of *PhPrtPI* as described previously in Materials and Methods. Each point represents the average of two experiments.

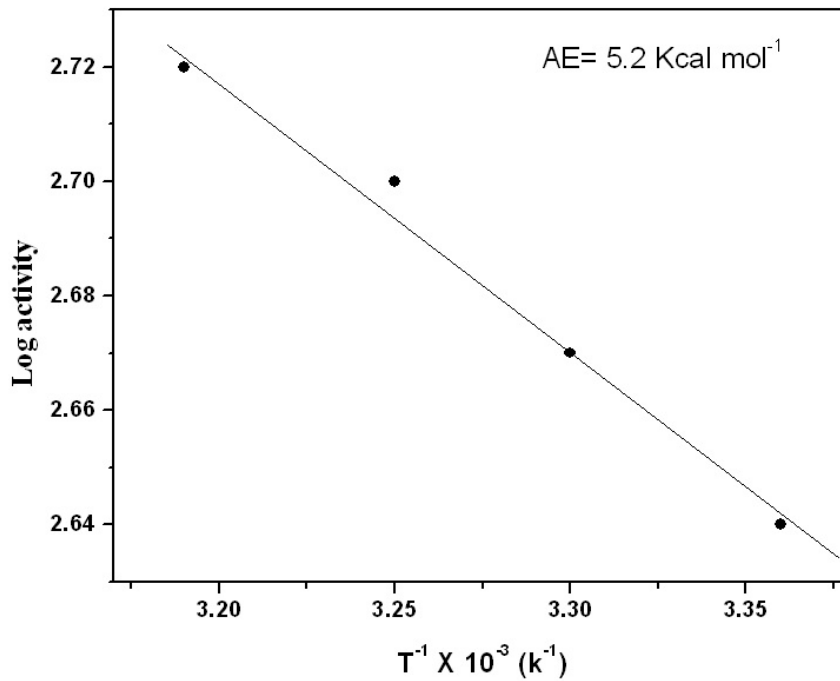


Fig. 5: Arrhenius plot, log activity versus $1/\text{Temperature}$ for *PhPrtPI* Reaction mixture was contained in 1.0 ml: 200 mM Tris-HCl buffer, pH 8.5, 2 mg azocasein and 45 units of enzyme. The reaction mixtures were incubated at different temperatures ranging from 25 to 40 °C. Each point represents the average of two experiments.

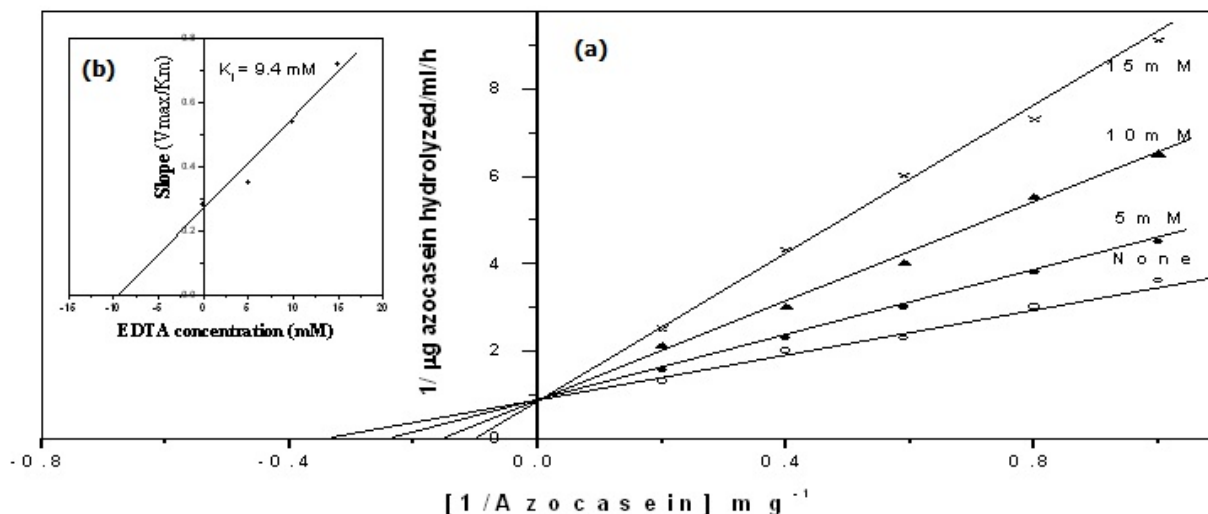


Fig. 6: (a), Inhibition study of *PhPrtPI* with EDTA. Plots of reciprocal initial velocities versus reciprocal concentrations of azocasein. Reaction mixtures contained in 1.0 ml: 200 mM Tris-HCl buffer, pH 8.5, azocasein and EDTA were added in the concentrations indicated. Each point represents the average of two experiments. (b), Determination of the inhibition constant (K_i) of EDTA as the competitor of azocasein for *PhPrtPI*.

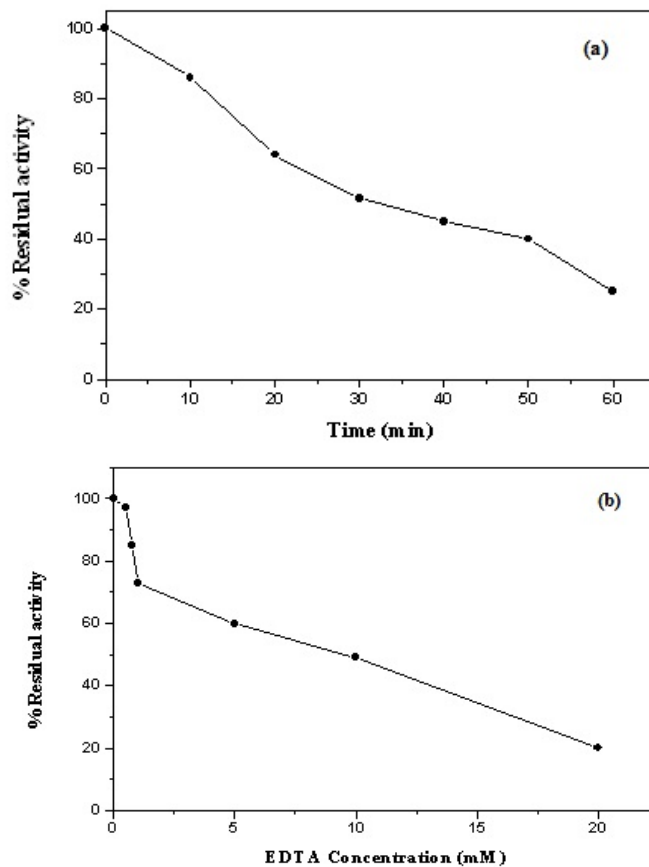


Fig. 7: (a): The effect of different EDTA concentration (b) and time incubation on activity of *PhPrtPI*. Activity in absence of EDTA was taken as 100%. The residual activity was measured using azocasein as substrate. Each value represents the average of two experiments.

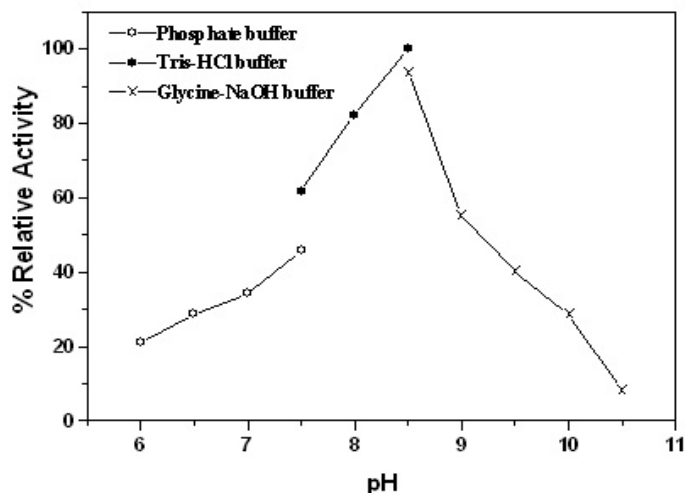


Fig 8: pH Optimum of *PhPrtPI* using azocasein as substrate. Each point represents the average of two experiments.

increasing the concentration of sulfhydryl reagents. Similar results, inhibition of metalloproteases by sulfhydryl reagents, have been observed for bacterial proteases including fish pathogen *Y. ruckeri*^[44], *F. psychrophilum*^[45], *P. luminescens* ssp. *laumondii* strain Brecon^[35,36] and *X. nematophila* BA2^[38]. Inhibition of *PhPrtPI* by sulfhydryl reagents suggested that disulfide bonds could be important in maintaining the molecular conformation required for the enzyme activity as reported by Secades and Guijarro^[44] and Secades *et al.*^[45]. Thereafter, Marokhazi *et al.*^[35] suggested that the strong inhibition of *P. luminescens* ssp. *laumondii* strain Brecon by thiol reducing agents indicate a role for thiol group (s) in enzymatic activity. Recently, Marokhazi *et al.*^[36] have confirmed such hypothesis and concluded that the inhibitory effects of disulfide bridge-reducing agents was probably not the removal of the metal ion but, perhaps, was due to a binding to the catalytic metal ion.

Ph Optimum: Metalloproteases can be further subdivided into neutral or alkaline metalloproteases on the basis of their pH optima^[43,16,11]. In the present study *PhPrtPI* can be classified as an alkaline metalloprotease since the optimal activity was observed at pH 8.5 (Fig. 3). This result was congruent with that reported for purified enzymes from different species of *Photobacterium*^[5,18,7,9] and *Xenorhabdus*^[30,38] and from fish pathogen *Y. ruckeri*^[45]. The maximal activity of *PhPrtPI* herein is 0.5 higher than that estimated for *P. luminescens* strain Hm (pH 8.0)^[43]. While a neutral pH optimum (pH 6.5-7.0) has been estimated for metalloproteases purified from *X. nematophila* strain Breton^[10], *X. nematophilus*^[12] and *P. luminescens* ssp. *laumondii* strain Brecon^[35].

It has been also reported that proteolysis of the insect cadavers should cause an alkaline shift in the pH of the insect cadavers^[43,20]. This shift occurs, as determined in the present study by the change in color of the *G. mellonella* cadavers from yellow to red. This change is due to a pigment produced by the bacterium *Photobacterium* sp. strain EK1 in the present study that undergoes a pH-sensitive color shift from yellow at near-neutral pH to red near pH 9 as reported by Richardson *et al.*^[42,43,53] Taking into consideration the initial pH of the insect hemolymph is 6.5^[17,43].

Substrate Specificity: The ability of *PhPrtPI* substrates revealed that the enzyme was active on fibrin, hemoglobin, gelatin, albumin and collagen with relative activity rates of 75, 74.3, 67.1, 54.6 and 37.1% respectively comparing with casein as 100% hydrolysis. (Table3). The Km values for *PhPrtPI* alkaline protease using such protein substrates in addition to casein were estimated to be 8.3, 11.1, 12.5, 15.4, 25 and 5 mg substrate/ml respectively with Vmax 1.7, 1.7, 1.6, 2.43, 0.003 and 1.8X10³ μg substrate hydrolyzed/h respectively (Table4). Similarly, different microbial metalloproteases have specificity towards natural protein substrates with different degrees^[51,13,9,28,]. Generally, they have a broad range of peptide and protein substrates^[37]. The highest activity of *PhPrtPI* towards casein, fibrin, hemoglobin but with small extent towards collagen is in agreement with those reported for microbial metalloproteases from entomopathogenic bacteria *P. temperata* strain K122^[52,7], strain W14^[6], *X. nematophila* strain Breton^[10], *X. nematophila* BA2^[38], and for fish pathogen *F. psychrophilum*^[45]. The lowest ability of *PhPrtPI* to degrade collagen suggests a degree of specificity that might be physiologically important.

Table 2: Effect of different compounds, activators and inhibitors on the activity of *Photorhabdus* sp. alkaline protease PI.

Compound	Concentration(mM)	Inhibition(%)
None	---	0.0
Cysteine	1.0	62.8
	10.0	96.4
Dithiothreitol	1.0	64.7
	10.0	96.6
β - mercaptoethanol	1.0	52.9
	10.0	98.1
Metalloprotease inhibitor		
EDTA	1.0	47.2
	10.0	52.9
	20.0	80
EDTA (20mM) + Ca ²⁺	10	17.5
	15	13
	20	8.5
1,10 Phenanthroline	1.0	0.0
	10.0	10
	20.0	25.4
Cysteinyl protease inhibitor		
N-Ethylmaleimide	1.0	5.9
	10.0	13.0
Iodoacetic acid	1.0	5.7
	10.0	6.2
p-CMB	1.0	5.7
	10.0	7.6
Serine protease inhibitor		
Soybean trypsin I	1.0 mg	0.0
	5.0 mg	5.7
PMSF	1.0	0.0
	10.0	0.0
Leupeptin	1.0	1.5
	10.0	2.6
Aspartyl protease inhibitor		
Pepstatin A	1.0	0.0
	10.0	5.4

The enzyme was preincubated for 15 min at 37°C with the listed compounds individually at the final concentration indicated prior to substrate addition. Activity in absence of compounds was taken as 100%. Each value represents the average of two experiments.

Bacterial pathogens produce various proteases, which contribute to the microbial virulence by hydrolyzing biologically important proteins and peptides^[41,37]. However, we have no indication of the function of *PhPrtpI* herein *in vivo*. The broad substrate specificity of the enzyme towards different proteins referred to the ability of the bacterium *Photorhabdus* strain EK1 to digest some proteins of the insect hemolymph as reported by Bowen *et al.*^[7] and Cabral *et al.*^[9].

Table 3: Relative activity of *Photorhabdus* sp. EK1 alkaline protease PI towards different native protein substrates.

Protein substrate	Relative activity(%)
Casein	100
Albumin	54.6
Hemoglobin	74.3
Fibrin	75
Collagen	37.1
Gelatin	67.1

All assays were incubated for 2 h at 37 °C in 200 mM Tris-HCl buffer, pH 8.5, 45 units of enzyme and 2 mg substrate. Casein was taken as 100 % activity. Each value represents the average of two experiments.

Table 4: The kinetic properties of *Photorhabdus* sp. EK1 alkaline protease PI.

Substrates	K _m (mg protein/ml)	V _{max} × 10 ³
Casein	5	1.8
Albumin	15.4	2.43
Hemoglobin	11.1	1.7
Fibrin	8.3	1.7
Collagen	25	0.003
Gelatin	12.5	1.6

The hydrolysis of such proteins may provide nutritional factors to the associated nematode necessary for its complete development and reproduction inside the infected insect cadaver^[10,15,7,9,36]. Other possible function of alkaline metalloprotease for *Photorhabdus* sp. strain EK1 herein including the inactivation of non-specific insect's defence mechanism that is could be postulated as reported by Caldas *et al.*^[10] and Marokhazi *et al.*^[36] Recently, Marokhazi *et al.*^[36] suggested that alkaline metalloprotease PrtA from *P. luminescens* ssp. *laumondii* strain Brecon participated in the establishment of infection inside an insect host.

Despite the broad substrate specificity of *PhPrtpI*, the bacterium carrying nematode goes through a complete life cycle within the insect cadavers and the IJs presumably are exposed to the bacterial proteases. The lowest ability of *PhPrtpI* to degrade collagen, the primary component of the nematode's cuticle^[34] would argue for the absence of a collagenase activity and the involvement of such activity in the destruction of the collagenous matrix of the nematode's cuticle could turn out.

Conclusion: In summary, here we characterized the second protease isoenzyme secreted. The *PhPrtpI* is an alkaline metalloprotease and it is Ca²⁺ dependent. Its substrate specificity strengthens the possibility that it is involved in degradation of insects tissue for providing nutrients to the associated nematodes, which is unable to grow on insect without a previous bioconversion of the insect cadavers by the symbiotic bacteria.

REFERENCES

1. Abd Rahman, R.N., L.P. Geok, M. Basri and A.B. Salleh, 2006. An organic solvent-stable alkaline protease from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. *Enz. And Microbial Technol.*, 39: 1484-1491.
2. Abu-Hatab, M., R.I. Sturat and R. Gaugler, 1998. Antibiotic resistance and protease production by *Photorhabdus luminescens* and *Xenorhabdus poinarii* bacteria symbiotic with entomopathogenic nematodes: variation among species and strains. *Soil Biol. Biochem.*, 30: 1955-1961.

3. Adinarayana, K., P. Ellaiah and DS. Prasad, 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS. Pharm. Sci. Tech., 5(4): E56.
4. Akhurst, R.J., 1980. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. J. Gen. Microbiol., 121: 303-309.
5. Bowen, D.J. and J.C. Ensign, 1998. Purification and characterization of a high-molecular weight insecticidal protein complex produced by the entomopathogenic bacterium *Photorhabdus luminescens*. Appl. Environ. Microbiol., 64: 3029-3035.
6. Bowen, D.J., M. Blackburn, T. Rocheleau, C. Grutzmacher and R.H. French-Constant, 2000. Secreted proteases from *Photorhabdus luminescens*: separation of the extracellular proteases from the insecticidal Tc toxin complexes. Insect Biochem. Mol. Biol., 30: 69-74.
7. Bowen, D.J., T.A. Rocheleau, C. Grutzmacher, L. Meslet, M. Valens, D. Marble, A. Dowling, R.H. French-Constant, and M.A. Blight, 2003. Genetic and biochemical characterization of PrtA, an RTX-like metalloprotease from *Photorhabdus*. Microbiol., 149: 1581-1591.
8. Brock, F.M., C.W. Forsberg, and J.G. Buchanan-Smith, 1982. Proteolytic activity of human microorganisms and effect of proteinase inhibitors. Appl. Environ. Microbiol., 44: 561-569.
9. Cabral, C.M., A. Cherqui, A. Pereira and N. Simoes, 2004. Purification and characterization of two distinct metalloproteases secreted by the entomopathogenic bacterium *Photorhabdus* sp. Strain Az29. Appl. Environ. Microbiol., 70: 3831-3838.
10. Caldas, C., A. Cherqui, A. Pereira and N. Simoes, 2002. Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immune suppression. Appl. Environ. Microbiol., 68: 1297-1304.
11. Cha, M., J.R. Park and K.Y. Yoon 2005. Purification and characterization of an alkaline serine protease producing angiotensin I-converting enzyme inhibitory peptide from *Bacillus* sp. SS103. J. Med. Food, 8: 462-8.
12. Chae, Y.R. and K.G. Ryu, 2004. Partial purification and characterization of an extracellular protease from *Xenorhabdus nematophilus*, a symbiotic bacterium isolated from an entomopathogenic nematode, *Steinernema glaseri*. Biotech. and Bioprocess Engineering, 9: 379-382.
13. Clark, D.J., S.J. Hawrylik, E. Kavanagh and D.J. Ophem, 2000. Purification and characterization of a unique alkaline elastase from *Micrococcus luteus*. Protein Exp. Purif., 18: 46-55.
14. Dabron, P.J., N. Waterfield, M.A. Blight and R.H. French-Constant, 2001. Measuring virulence factor expression by the pathogenic bacterium *Photorhabdus luminescens* in culture and during insect infection. J. Bacteriol., 183: 5834-5839.
15. Dowds, B.C.A. and A. Peters, 2002. Virulence mechanisms. In: Gaugler, R. (Ed.), Entomopathogenic Nematology, pp. 79-98. CABI, Wallingford, UK.
16. Dumont, L., B. Verneuil, J. Wallach and R. Julien 1994. Purification and characterization of an alkaline elastase from *Myxococcus xanthus*. Eur. J. Biochem., 3: 775-782.
17. Dunphy, G.B., D.B. Morton, A. Kvopinski and J.M. Chadwick, 1986. Pathogenicity of lipopolysaccharide mutants of *Pseudomonas aeruginosa* for larvae of *Galleria mellonella*: bacterial properties associated with virulence. J. Invertebr. Pathol., 47: 48-55.
18. Fischer-Le Saux, M., V. Viillard, B. Brunel, P. Normand and E. Boemare, 1999. Polyphasic classification of the genus *Photorhabdus* and proposal of new taxa: *P. luminescens* subsp. *luminescens* subsp. nov., *P. luminescens* subsp. *akhurstii* subsp. nov., *P. luminescens* subsp. *laumondii* subsp. nov., *P. temperata* sp. nov., *P. temperata* subsp. *temperata* subsp. nov., and *P. symbiotica* sp. nov. Int. J. Syst. Bacteriol., 49: 1645-1656.
19. Forst, S., B. Dowds, N. Boemare and E. Stackebrandt, 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annu. Rev. Microbiol., 51: 47-72.
20. Forst, S. and D. Clarke, 2002. Bacteria-nematode symbiosis. In: Gaugler, R. (Ed), Entomopathogenic Nematology, pp. 57-77. CABI, Wallingford, UK.
21. Fricke, B., V. Parchmann, K. Kruse, P. Rucknagel, A. Schierhorn and S. Menge, 1999. Characterization and purification of an outer membrane metalloproteinase from *Pseudomonas aeruginosa* with fibrinogenolytic activity. Biochem. Biophys. Acta., 1454: 236-250.
22. Gaugler, R. and R. Han, 2002. Production technology, In: Gaugler, R. (Ed), Entomopathogenic Nematology, pp. 289-310. CABI, Wallingford, UK.
23. Gomori, G., 1955. Preparations of buffers for use in enzyme studies. In: Colowick, S.P. and Kaplan, N.O. (Eds), Methods in Enzymology. pp. 138-146, Academic Press, New York.

24. Grewal, P.S., R.U. Ehlers and D.I. Shapiro-Ilan, 2005. Nematodes as biocontrol agents. CABI Publishing, Wallingford, UK. pp. 505-515.
25. Gupta, A., I. Roy, S.K. Khare and M.N. Gupta, 2005. Purification and characterization of a solvent stable protease from *Pseudomonas aeruginosa* PseA. J. Chromat., 1069: 155-161.
26. Held, K.G., C.N. LaRock, D.A. D'Argenio, C.A. Berg and C.M. Collins, 2007. A metalloprotease secreted by the insect pathogen *Photorhabdus luminescens* induced melanization. Appl. Environ. Microbiol., pp. 7622-7628.
27. Huang, Q., Y. Peng, H. Wang and Y. Zhang, 2003. Purification and characterization of an extracellular alkaline protease with dehairing function from *Bacillus pumilus*. Curr. Microbiol., 46: 169-73.
28. Huang, X., B. Tian, Q. Niu, J. Yang, L. Zhang and K. Zhang, 2005. An extracellular protease from *Brevibacillus laterosporus* G4 without parasporal crystals can serve as a pathogenic factor in infection of nematodes. Research in Microbiol., 156: 719-727.
29. Kazan, D., A.A. Denizci, M.N. Oner and A. Erarslan, 2005. Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42. J. Indian Microbiol. Biotech., 32: 335-344.
30. Kucera, M., and Z. Mracek, 1989. Partial purification and properties of proteolytic enzymes of Steinernematode nematodes pathogenic for *Galleria mellonella* larvae. Acta Entomol. Bohemoslov., 86: 342-348.
31. Kumar, C.G., 2002. Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. Lett. Appl. Microbiol., 34: 13-7.
32. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
33. Lewis, E.E., J. Campbell, C. Griffin, H. Kaya and A. Peters, 2006. Behavioral ecology of entomopathogenic nematodes. Biol. Cont., 38: 66-79.
34. Li, H.Y., G.D. Yang, H.R. Shu, Y.T. Yang, B.X. Ye, I. Nishida and C.C. Zheng, 2006. Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (*Vitis amurensis* Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. Plant Cell Physiol., 47: 154-319.
35. Marokhazi, J., K. Lengyel, S. Pekar, G. Felfoldi, A. Patthy, L. Graf, A. Fodor and I. Venekei, 2004. Comparison of proteolytic activities produced by entomopathogenic *Photorhabdus* bacteria: strain- and phase-dependent heterogeneity in compositin and activity of four enzymes. Appl. Environ. Microbiol., 70: 7311-7320.
36. Marokhazi, J., N. Mihala, F. Hudecz, A. Fodor, L. Graf and I. Venekei, 2007. Cleavage site analysis of a serralysin-like protease, PrtA, from an insect pathogen *Photorhabdus luminescens* and development of a highly sensitive and specific substrate. FEBS Journal, 274: 1964-1956.
37. Miyoshi, S. and S. Shinoda, 2000. Microbial metalloproteases and pathogenesis. Microbes and Infec., 2: 91-98.
38. Mohamed, M.A., M.A. Hussein, 2007. Purification and characterization of an alkaline protease produced by the bacterium *Xenorhabdus nematophila* BA2, a symbiont of entomopathogenic nematode *Steinernema carpocapsae*. Res. J. Agric. Biol. Sci., pp. 510-521.
39. Mohamed, M.A., H.H. El Hendawy, A.M. Ghazy, A.M. Abd El Aziz, 2008. Purification and characterization of an alkaline metalloprotease secreted by the bacterium *Photorhabdus* sp. EK1, a symbiont of entomopathogenic nematode *Heterorhabditis bacteriophora*. Comp. Biochem. Physiol. Part B. In press.
40. Morgan, J.A.W., M. Sergeant, D. Ellis, M. Ousley and P. Jarret, 2001. Sequence analysis of insecticidal genes from *Xenorhabdus nematopjilus* PMFI296. Appl. Environ. Microbiol., 67: 2062-2069.
41. Nagase, H. and J.F.J. Woessner, 1999. Matrix metalloproteinases. J. Biol. Chem., 274: 21491-21494.
42. Richardson, W.H., T.M. Schmidt and K. Neelson, 1988. Identification of an anthraquinone pigment and a hydroxystilbene antibiotic from *Xenorhabdus luminescens*. Appl. Environ. Microbiol., 54: 1602-1605.
43. Schmidt, T.M., B. Bleakley and K.H. Neelson, 1988. Characterization of an extracellular protease from the insect pathogen *Xenorhabdus luminescens*. Appl. Environ. Microbiol., 54: 2793-2797.
44. Secades, P. and J.A. Guijarro, 1999. Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. Appl. Environ. Microbiol., 65: 3969-3975.

45. Secades, P., B. Alvarez and J.A. Guijarro, 2001. Purification and characterization of a psychrophilic, calcium-induced, growth-phase-dependent metalloprotease from the fish pathogen *Flavobacterium psychrophilum*. *Appl. Environ. Microbiol.*, 67: 2436-2444.
46. Secades, P., B. Alvarez. and J.A. Guijarro, 2003. Purification and properties of a new psychrophilic metalloprotease (Fpp2) in the fish pathogen *Flavobacterium psychrophilum*. *Fed. Eur. Microbiol.*, 54: 273-279.
47. Sicard, M., J. Hinsinger, N. Le Brun, S. Pages, N. Boemare, C. Moulia, 2006. Interspecific competition between entomopathogenic nematodes (*Steinernema*) is modified by their bacterial symbionts (*Xenorhabdus*). *BMC Evol Biol.*, Sep 5:6-68.
48. Takeuchi, S., T. Kinoshta, T. Kaidoh and N. Hashizume, 1999. Purification and characterization of protease produced by *Staphylococcus aureus* isolated from a diseased chicken. *Vet. Microbiol.*, 67: 195-202.
49. Thangam, E.B. and G.S. Rajkumar, 2002. Purification and characterization of alkaline protease from *Alcaligenes faecalis*, *Biotech. Appl. Biochem.*, 35: 149-154.
50. Tomarelli, R.M., J. Charney and M.L. Harding, 1949. The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. *J. Lab. Clin. Med.*, 34: 428-433.
51. Turkiewicz, M., E. Gromek, H. Kalinowska, and M. Zielinska, 1998. Biosynthesis and properties of an extracellular metalloprotease from the Antarctic marine bacterium *Sphingomonas paucimobilis*. *J. Biotech.*, 70: 53-60.
52. Waterfield, N., A. Dowling, S. Sharma, P.J. Daborn, U. Potter and R.H. French-Constant, 2001. Oral toxicity of *Photorhabdus luminescens* W14 toxin complex in *Escherichia coli*. *Appl. Environ. Microbiol.*, 67: 5017-5024.
53. Webster, J., G. Chen, K. Hu and J. Li, 2002. Bacterial metabolites, In: Gaugler, R. (Ed), *Entomopathogenic Nematology*, pp. 99-114. CABI, Wallingford, UK.
54. Woodring J.L. and H.K. Kaya, 1988. *Steinernematid and Heterorhabditid nematodes: a handbook of techniques*. Southern Cooperative Series Bulletin 331, Arkansas Agricultural Experiment Station, Fayetteville, U.S.A.
55. Zeng, R., R. Zhang, J. Zhao and N. Lin, 2003. Cold-active alkaline protease from the psychrophilic bacterium *Pseudomonas* strain DY-A: enzyme purification and characterization. *Extremophiles.*, 7: 335-7.