Purification and Characterization of an Alkaline Protease Produced by the Bacterium
Xenorhabdus nematophila BA2, a Symbiont of Entomopathogenic Nematode Steinernema carpocapsae

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Abstract: Xenorhabdus nematophila, a bacterium pathogenic for insects associated with the nematode Steinernema carpocapsae, secretes proteases during growth. As the bacterial population increased, the proteolytic activities increased and reached its maximum level at the stationary phase, after 48 h of inoculation. A reduction in the enzymatic activity was recorded by increasing the time of inoculation. The isoenzyme pattern of proteolytic activities revealed that one slow and two fast isoenzymes were released during bacterial growth. The major isoenzyme, X. nematophila PAII, was purified to homogeneity in a three-step procedure involving ammonium sulfate fractionation, gel filtration and ion-exchange chromatography. The purified enzyme had a specific activity of 295 units/mg protein with purification fold 7.7 over crude extract. A molecular weight of 39 kDa was estimated for both the native and denatured enzyme, suggesting that the enzyme is monomeric. The enzyme had characteristics of a cold-adapted protein, it was more active in the range of 15 to 30°C and had an optimum activity at 30°C. The activation energy for the hydrolysis of azo casein was determined to be 16.1 kcal/mol. Temperatures above 30°C have deleterious effect on the enzyme activity and stability. The enzyme activity was totally abolished by 1 mM EDTA and 1,10-phenanthroline, but not affected by cysteine, serine and aspartyl protease inhibitors. X. nematophila PAII had an optimum pH of 8.5 and classified as an alkaline metalloprotease. Its substrate specificity strengthens the possibility that it is 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 bacteria. Its biochemical characteristics were compared with those previously reported for different species of animal pathogenic bacteria.

Keywords: alkaline, bacteria, characterization, entomopathogenic nematodes, Xenorhabdus, protease, purification.

INTRODUCTION

The bacteria of the genus Xenorhabdus are gram-negative bacteria that form a mutualistic symbiosis with entomopathogenic nematodes (EPNs) from the family Steinernematidae. These bacteria are carried in an intestinal vesicle of the infective-nonfeeding-third-stage juveniles (IJs). The two partners, acting in concert, are voracious pathogens to a wide variety of insect larvae and are currently used as biological control agents. The IJ locates a susceptible insect victim, enters the hemocoel and releases its charge of bacteria. The bacteria actively multiply avoiding the host defenses and causing an acute disease conditions that are followed by insect death within 48h.\textsuperscript{[10,20,5,9,48]}

The set of mechanisms by which these bacteria are able to circumvent the host defense systems and cause insect death is frequently associated with the extracellular molecules produced by Xenorhabdus species.\textsuperscript{[6,15,3,5,47]}

All the bacteria of the genus Xenorhabdus spontaneously produce two distinct physiological states phase I and phase II. Phase I cells are associated only with the IJs that naturally parasitize insects\textsuperscript{[5,12,13,11,47]}. It produce a wide range of toxins and a complex set of extracellular enzymes including lipases, phospholipases, chitinases, phosphatases 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 inside the insect cadavers. While these properties are greatly reduced in phase II\textsuperscript{[10,18,20,11,45]}. Proteases represent important part of
the extracellular enzymes, although their role in the virulence process is yet unclear.

Extracellular alkaline proteases produced by different species of *Photorhabdus*, a symbiont of the EPNs *Heterorhabditis* species [6,34,5,11] and *X. nematophila* strain Kraussei, a symbiont of the *Steinernema carpocapsae* [23], have been purified, characterized and classified as alkaline metalloproteases. They have also been purified and characterized from several animal pathogenic bacteria including fish pathogens *Yersinia ruckeri* [44] and *Flavobacterium psychrophilum* [46] and from human pathogenic bacteria *Pseudomonas aeruginosa* [15], *Serratia marcescens* [13] and *Bacteroides fragilis* [13]. Recently, they have also been purified and characterized from different species of *Bacillus* [27,42,34], *Pseudomonas* [21] and *Stenotrophomonas* [35].

Bacterial proteases are mainly involved in providing peptide nutrients for the microorganisms. In fact, some authors regard bacterial proteases as the main virulence factors present among the extracellular factors [44,36,46,16]. Although extracellular proteases produced by entomopathogenic bacteria play a role in insect toxicity, their physiological importance is highly conflicting. Certain literatures acknowledge that proteases might have a role in insect toxicity by analogy with proteases produced by other pathogens [43,25]. Whereas other authors claim that these proteases are not toxic to the insects, neither by injection nor by oral feeding [49]. These conflicting literatures prompted us to carry out the present study.

This study has focused on the estimation of extracellular alkaline proteases during bacterial growth of the bacterium *X. nematophila* BA2, a symbiont of the EPN *Steinernema carpocapsae* BA2 that has been previously identified and characterized by Hussein and Abo-Elsoud [24]. We report here also the purification and characterization of the predominant isoenzyme of an alkaline protease from the growth medium of the highest proteolytic activity. The present study aims also to compare its physical and biochemical properties with those previously purified from different animal pathogenic bacteria. This information is leading to address its significant role/s in virulence and to understand the molecular basis of symbiosis and insect toxicity to improve the use of such symbiotic complex as a biocontrol agent against insect pests.

**MATERIALS AND METHODS**

**Nematode Source and Isolation of Bacteria:** The IJs of the EPN *S. carpocapsae* BA2 were isolated from RasSedr, South Saini, Egypt by Hussein and Abo-Elsoud [24] 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 [57]. 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. The bacterium *X. nematophila* strain BA2, a symbiont of the nematode *S. carpocapsae* BA2, was isolated according to Caldas et al. [12] and Cabral et al. [11]. The IJs were surface sterilized for 2 min in 0.1% sodium hypochlorite, washed in sterile distilled water several times, cut into several pieces by blades and transferred to a petri dish containing 5 ml of Luria-Bertani (LB) medium plates (1% pepton, 0.5% yeast extract, 0.5% NaCl). The plates were incubated at 28°C for 24 h and single suitable colony was spread on medium plates of nutrient bromothymol blue (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). The bacteria colonies used in this study were phase I variant because they showed an olive green color on NBTA medium [40] and maintained in −80°C.

**Proteolytic Activity During Bacterial Growth:** The growth curve of *X. nematophila* strain BA2 cultures were obtained by monitoring the optical density at 600 nm according to Caldas et al. [12]. For this purpose, 250-ml flasks contain 50 ml of LCM were inoculated with 0.2 ml of phase I bacterial colonies from the stock culture. The flasks were incubated for different time ranging from 0 to 120 h at 28°C in an orbital shaker at 150 rpm and three replicates were prepared. To monitor the protease activity during bacterial growth, 5ml aliquots were sampled at different time cut into several pieces by blades and intervals and centrifuged at 12,000Xg for 10 min at 4°C to remove the bacterial cells, and the supernatants were stored at -20°C until used for enzymatic analysis. For purification of an alkaline protease, cultures were obtained in flasks as perviously described and incubated for 48h.

**Enzyme Assay:** The proteolytic activity was determined as described by Tomarelli et al. [51] and modified by Cabral et al. [41]. 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. 

Protein was determined either by measuring the absorbance at 280 and 260 nm or by the method of Bradford using bovine serum albumin as a standard.

**Buffers:** Buffers were prepared according to Gomori and the final pH was confirmed with a pH meter.

**SDS-PAGE Zymogram:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% (w/v) acrylamide copolymerized with 0.1% gelatin according to Cabral et al. Conditions for electrophoresis were essentially as described by Laemmli under nondenaturing conditions and run at 100 V. Following electrophoresis, the gel was washed for 30 min in 50 mM Tris-HCl buffer, pH 8.5 containing 2.5% Triton X-100, with gentle agitation in order to remove the excess of SDS. Then, the gel was incubated overnight at the same buffer. Zones of proteolysis were detected by staining overnight with Coomassie Brilliant Blue R 250. Proteolytic activity appeared as clear bands on a blue background.

**Purification of an Alkaline Protease:** Unless otherwise stated all steps were performed at 4-7°C.

**Preparation of Crude Extract:** The purification scheme entailed starting with 100 ml of 48-h culture broth of *X. nematophila* strain BA2. The culture broth was centrifuged at 12,000Xg for 10 min at 4°C, and subjected to ammonium sulfate precipitation.

**Ammonium Sulfate Fractionation:** Solid ammonium sulfate was added slowly to the cell free supernatant with continuous stirring and cooling to a final concentration of 40% (w/v). The mixture was stirred for 15 min and the precipitate (AI) was collected by centrifugation at 10,000Xg for 10 min with cooling. Ammonium sulfate was increased in the supernatant up to 85% saturation with stirring and cooling followed by centrifugation to collect the second precipitate (AII). The precipitate (AII) was dissolved in 2 ml of 20 mM Tris-HCl buffer, pH 8.0.

**Sephacryl S-200 Chromatography:** The precipitate (AII) was applied on the top of a Sephacryl S-200 column (95 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0, developed with the same buffer at a flow rate of 6 ml/h according to Kucera and Mracek.

**DEAE-cellulose Chromatography:** The pooled active fractions (PA) of the Sephacryl S-200 column were applied directly to a DEAE-cellulose column (10 x 1.6 cm i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The adsorbed material was eluted with a stepwise gradient NaCl ranging from 0.0 to 0.5 M prepared in the same buffer at a flow rate of 60 ml/h and 5 ml fractions were collected. Protein fractions exhibiting protease activity were eluted with 0.1 and 0.2 M NaCl, respectively and designated as PA1 and PAII, respectively according to their elution order.

**Polyacrylamide Gel Electrophoresis (PAGE):** Electrophoresis under non-denaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis using a Tris-glycine buffer, pH 8.3 to examine the final enzyme preparation for its purity. Protein bands were stained with Coomassie Brilliant Blue R-250.

**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 (29 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), alcohol dehydrogenase (150 kDa) and ß-amylase (200 kDa). Dextran blue (200000) was used to determine the void volume (Vo). Subunit molecular weight was estimated by SDS-polyacrylamide gel electrophoresis. SDS-denatured bovine serum albumin (67 kDa), ovalbumin (43 kDa), glyceraldehyde 3-P-dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa) and α-lactalbumin (14.2 kDa) were used as marker proteins for the calibration curve.

**Effect of pH on Enzyme Activity:** The effect of pH on the activity of *X. nematophila* PAII 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.

**Effect of Temperature on Enzyme Activity and Stability:** To examine the effect of temperature on the activity of *X. nematophila* PAII, the complete enzyme reaction mixtures were incubated at different temperatures ranging from 15 to 55°C. For estimating the effect of temperature on the enzyme stability, the enzyme was preincubated for 30 min at different temperatures ranging from 15 to 55°C, followed by cooling and measuring the remaining activity as described previously.

**Inhibition Studies:** To determine the class, to which the *X. nematophila* PAII belongs, azocaseinolytic
activity was measured in presence of several inhibitors and activators. The enzyme was incubated with phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), ethylenediaminetetraacetic acid (EDTA), leupeptin, pepstatin A, N-ethylmaleimide (NEM), 1,10-phenanthroline, dithiothreitol (DTT) and cysteine individually for 30 min at room temperature and followed by measuring the remaining activity.

**The Substrate Specificity:** The substrate specificity of *X. nematophila* PAII 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.

### RESULTS AND DISCUSSION

After the release of entomopathogenic bacteria into the insect hemocoel by the EPNs, the bacteria appear to rapidly overcome the insect’s defense mechanisms and kill the insect. During growth within the insect, the bacterium is thought to release a variety of compounds including lipases, antibiotics, lipopolysaccharides and proteases. Previous studies on the extracellular proteases have been equivocal as to their role in insect toxicity. Thus, some authors have directly implicated them as toxic components, while others have suggested that they play a specific role in attacking antibacterial defense system of the insect. Still others have found no correlation between extracellular proteases and insect toxicity. In the present study we are interested in estimating alkaline proteases produced by the bacterium *X. nematophila* BA2, a symbiont of the EPN *S. carpocapsae*, and purifying the major isoenzyme to address its physiological role/s in the virulence process.

**Effect of Incubation Time on Bacterial Growth and Proteolytic Activity:** The growth curve of *X. nematophila* BA2 and the proteolytic activity recovered in the culture media were monitored for 120 h to determine the optimal culture time that had the maximal enzyme activity. The results revealed that *X. nematophila* BA2 grow in liquid medium (LCM) until reached the stationary phase after 48 h of incubation (Fig. X1).

As the bacterial population increase, the enzyme activity increase and reached the maximal level at the stationary growth phase of the bacteria, at 48 h postinoculation (8.6 units/ml). A reduction in enzymatic activity was observed by increasing the incubation time where, 4.8 units/ml was recorded after 120 h of incubation.

![Fig. 1: Release of proteolytic activity in the culture broth during growth of *X. nematophila* BA2. Samples of culture medium supernatants were collected at different times and proteolytic activities were estimated using azocasein as substrate. Proteolytic activity (x) and bacterial growth (o).](image)

**Fig. 2:** Zymography of SDS-PAGE copolymerized with gelatin under non-denaturing conditions for alkaline proteases produced by the bacterium *X. nematophila* BA2 in the culture supernatants at different incubation time.

Alkaline proteases have been estimated in different species of entomopathogenic bacteria of the genera *Xenorhabdus* and *Photobacterium* that are live in symbiosis with steinernematid and heterorhabditid nematodes, respectively. The reduction in the proteolytic activity produced by *X. nematophila* BA2 during the stationary phase was in agreement with that recorded for *Photobacterium* sp.
Fig. 3: A typical elution profile for the chromatography of X. nematophila alkaline protease on Sephacryl S-200 column (95 x 1.6 i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0 at a flow rate of 6 ml/h and 5 ml fraction. Absorbance at 280 nm (o____o) and protease activity (x__x).

Fig. 4: A typical elution profile for the chromatography of pooled active fractions of Sephacryl S-200 (PA) of X. nematophila alkaline protease on DEAE-cellulose column (10 x 1.6 i.d.) previously equilibrated with 20 mM Tris-HCl buffer, pH 8.0. Elution was carried out with different concentrations of NaCl in buffer, at a flow rate of 60 ml/h and 5 ml fractions. Absorbance at 280 nm (o____o) and protease activity (x____x).

strain Az29[11], P. temperata strain K122[9], X. nematophila strain Breton[12] and fish pathogen Y. rucker[24]. On the contrary, no significance reduction in enzymatic activity produced by P. luminescens strain Brecon/1 during the stationary phase[12] was observed.

The current study revealed that during the growth of the bacterium X. nematophila BA2, SDS-PAGE copolymerized with gelatin (Fig. 2) showed three different isoenzymes of proteases P3, P2 and P1, with Rf values 0.33, 0.79 and 0.9, respectively were released in the culture supernatants. While a protease P1 appeared in the growth media of 6, and 24 h postinoculation, two additional proteolytic bands P2 and P3 were appeared in the zymogram at 48 and 54 h postinoculation. The isoenzyme band of P3 is more intense than P2 and P1. During the stationary phase, the proteolytic activity reduced and only protease P1 persisted at 96 and 120 h of postinoculation.

A variable number of protease isoenzymes have been detected in different strains of Photorhabdus and Xenorhabdus species at different incubation time[12,55,11]. While a single isoenzyme was detected in the supernatant of the growth medium of P. luminescens strain Hm after 17 day of inoculation[41], two isoenzymes were produced by X. nematophila strain Breton[13] and X. nematophila strain Kraussei[28] after 24 and 48 h postinoculation, respectively. Three distinct isoenzymes were isolated from the culture
media of *P. luminescens* W14[12] and *P. luminescens* HP88[40] at 72 h postinoculation. Marokhazi *et al.*[32] detected also proteolytic activity in the culture supernatants of a different strains of *P. luminescens* and *P. temperata* and the number of the isoenzyme bands was found to be ranged from 1 to 3 that are depend on the time of incubation. Thus, we can concluded that the variation in the enzymatic level of alkaline proteases produced by *X. nematophila* BA2, the number of the isoenzyme bands and the timing of protease production may be attributed to the presence of a protease inhibitor that is suppressed the proteolytic activity through the formation of an enzyme-inhibitor complex as reported by Wee *et al.*, Forst and Clarke[18], Valens *et al.*[32] and Marokhazi *et al.*,[32]. This assumption was confirmed by Wee *et al.*,[36] where a partial purification and characterization of a protease inhibitor from the entomopathogenic bacterium *P. luminescens* HP88 had been reported.

An interesting question is the physiological role that *X. nematophila* BA2 proteases may play. An answer would require enzyme purification for the major isoenzyme, enzymatic characterization and identification the substrate specificity and determine the protease family to which the major isoenzyme belongs. From the above results, purification and characterization of the major isoenzyme of an alkaline protease was restricted from the culture medium of the highest proteolytic activity (48-h postinoculation).

### Purification of an Alkaline Protease:

The predominant isoenzyme of an alkaline protease was purified from *X. nematophila* BA2 from the culture broth of 48-h incubation. Results of the purification steps were summarized in Table 1. Most of the enzymatic activity (80%) was recovered in the second ammonium sulfate fraction (AII). Upon chromatography of ammonium sulfate fraction on Sephacryl S-200, three isoenzymes of alkaline proteases were resolved where *X. nematophila* PA is the major isoenzyme (Fig. 3). Chromatography of the major pooled active fractions of Sephacryl S-200 (PA) on DEAE-cellulose revealed that two isoenzymes PAI and PAII were eluted with 0.1 and 0.2 M NaCl, respectively (Fig. 4). The enzyme purity of *X. nematophila* PAI was assessed by electrophoresis, where the DEAE-cellulose fraction was resolved as a single band of protein to confirm the homogeneity (Fig. 5).

*X. nematophila* PAII exhibited a specific activity of 295.0 units/mg protein which represented 7.7-fold purification. The specific activity of *X. nematophila* PAII is 1.6, 3 and 8.1 times higher than that recorded for neutral metalloprotease produced by *X. nematophila* strain Breton[12], alkaline proteases from *X. nematophila* strain Krausse[21] and for *P. luminescens*[41], respectively. Although, it is 146-fold lower than that estimated for alkaline metalloprotease purified from *Photorhabdus* sp. strain Az29[11].

#### Characterization of *X. nematophila* PAII:

The molecular weight of *X. nematophila* PAII was found to be 39 kDa from the calibration curve of Sephacryl S-200 (Fig. 6). This value was confirmed by SDS-PAGE, where the enzyme migrated as a single protein band indicating that *X. nematophila* PAII is monomeric (Fig. 7).

The molecular weight (39 kDa) of *X. nematophila* PAII was congruent to that recorded for fish pathogen metalloprotease *Vibrio pelagius*[15] metalloproteases from different strains of *Photorhabdus*[12], *P. luminescens* strain W14[10]. The molecular weight of *X. nematophila* PAII is comparable equal (41 kDa) to that estimated for *X. nematophila* strain Krausse[21]. However, it is less by 1.58-fold than that recorded for *X. nematophila* strain Breton[12].

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**Table 1:** Purification scheme of an alkaline protease from *X. nematophila* BA2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (Units)*</th>
<th>Protein (mg)</th>
<th>Specific activity (Units/mg)</th>
<th>Fold purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>813</td>
<td>21.1</td>
<td>38.5</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (AII)</td>
<td>650</td>
<td>11.3</td>
<td>57.5</td>
<td>1.5</td>
<td>80</td>
</tr>
</tbody>
</table>

Sephacryl S-200

| Pooled PA                      | 384               | 4.1          | 93.7                         | 2.4               | 47.2         |
| Pooled PB                      | 125               | 4.9          | 25.5                         | 0.7               | 15.4         |
| Pooled PC                      | 86                | 0.9          | 95.5                         | 2.5               | 10.6         |

DEAE-cellulose for PA

| PAI (0.1 M NaCl)                | 101               | 1.7          | 59.4                         | 1.5               | 12.4         |
| PAII (0.2 M NaCl)               | 36                | 0.8          | 295.0                        | 7.7               | 29.0         |

* One unit of proteolytic activity was defined as µg azocasein hydrolyzed per hour under standard assay conditions.

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The thermal stability of *X. nematophila* PAII showed that the enzyme is highly unstable where more than 90% loss in enzyme activity was observed upon incubation for 15 min at 50°C (Fig. 8). This result is congruent with that observed for purified neutral metalloprotease from *X. nematophila* strain Breton, where 90% of proteolytic activity was lost upon incubation for 10 min at 60°C[13]. Alkaline proteases from fish pathogens, *Flavobacterium psychrophilum*[44] and *Y. ruckeri*[44], that are commonly affected salmonides, are also thermolabile where the enzyme activities completely lost upon incubation for 30 min at 55°C. Miyoshi *et al.*, [37] reported that *Vibrio fluvialis* metalloprotease is also heat labile, where 40% reduction in enzymatic activity was recorded upon incubation for 20 min at 50°C and it was completely abolished at 75°C. On the contrary, alkaline proteases produced by *P. luminescens* W14 and *P. temperata* K12[13], *Alcaligenes faecalis*[39], *Bacillus clausii* GMBAE42[21] and *Pseudomonas aeruginosa*[22] are thermostable where no detectable loss in enzyme activities could be recorded upon incubation for 30 min at 50°C.

The activity of *X. nematophila* PAII increased by increasing temperature and the optimum reaction temperature is 30°C. From 35°C onwards, the activity declined rapidly and it was finally undetectable at 55°C (Fig. 8). The optimum temperature for proteolytic activity of *X. nematophila* PAII (30°C) is 7°C higher and lower than that estimated for purified proteases.
Fig. 9: Arrhenius plot, log activity of X. nematophila PAII versus 1/T. Reaction mixtures were incubated at different temperatures ranging from 15 to 30°C. Each point represents the average of two experiments.

Fig. 10: pH Optimum for X. nematophila PAII. Phosphate buffer (x–x), Tris-HCl buffer (o–o) and glycine-NaOH buffer (o–o). Each point represents the average of two experiments.

Table 2: Effect of different protease inhibitors (I) and activators (A) on the activity of X. nematophila PAII.

<table>
<thead>
<tr>
<th>Compound*</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Serine protease I</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Soybean trypsin I</td>
<td>5.0</td>
<td>9.7</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Serine/Cysteine protease I</td>
<td>10.0</td>
<td>13.1</td>
</tr>
<tr>
<td>Leupeptine</td>
<td>0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>1.0</td>
<td>11.7</td>
<td></td>
</tr>
<tr>
<td>Cysteinyl protease I</td>
<td>0.1</td>
<td>2.7</td>
</tr>
<tr>
<td>N- Ethylmaleimide</td>
<td>1.0</td>
<td>8.6</td>
</tr>
<tr>
<td>10.0</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>10.0</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>p- CMB</td>
<td>1.0</td>
<td>3.8</td>
</tr>
<tr>
<td>10.0</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>Aspartyl protease I</td>
<td>0.01</td>
<td>2.3</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>0.1</td>
<td>11.5</td>
</tr>
<tr>
<td>Metalloprotease I</td>
<td>EDTA</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>80.7</td>
</tr>
</tbody>
</table>

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 the compounds was taken as 100%. Each value represents the average of two experiments. was carried out to determine the family at which the enzyme belongs. The results revealed that none of the serine protease and cysteine inhibitors inhibited X. nematophila PAII, nor did the aspartyl protease inhibitor (Table 2). However, the enzyme completely inhibited by the metal chelators EDTA (a general metalloprotease inhibitor) and 1,10-phenanthroline (a specific zinc metalloprotease inhibitor) at a concentration of 1 mM. These results suggested that the purified protease PAII from X. nematophila strain BA2 belong to a metalloprotease family.

Furthermore, no stimulatory effect was recorded upon incubation of X. nematophila PAII with low and high (0.1 and 5 mM) concentrations of cysteine and dithiothreitol (DTT). These results argue the absence of a thiol protease. Although, an inhibitory effects, 17.8 and 21% was observed upon incubation of the enzyme with 0.1 mM of cysteine and DTT,
respectively. This inhibitory effect increased by increasing the concentration of the reducing agents to 5 mM where 40.6 and 79.2% reduction in enzymatic activity was recorded, respectively. Similarly 70% inhibition was recorded for *Y. ruckeri* and 80% for *F. psychrophilum* metalloprotease upon incubation with 1 and 10 mM DTT, respectively. A non-metalloprotease from the entomopathogenic bacterium *P. luminescens* strain Brecon was strongly inhibited, 87.5 and 100%, by 1 mM cysteine and DTT[22]. Inhibition of *X. nematophila* PAII by DTT and cysteine suggests that disulfide bonds could be important in maintaining the molecular conformation required for activity as reported by Secades and Guijarro[48].

Metalloproteases can be further subdivided into neutral or alkaline metalloproteases on the basis of their pH optimum[41]. In the present study, *X. nematophila* PAII can be classified as an alkaline metalloprotease since the pH optimum is 8.5 (Fig. 10). This result was congruent with that reported for purified enzymes from different species of *Photobacteriurn*[9,17,5,11] and *X. nematophila* strain Kraussei[28]. It is in agreement also with those recorded for human pathogenic bacterium *P. aeruginosa*[49,52] and fish pathogen *Y. ruckeri*[48]. While a neutral pH optimum (7.0) has been estimated for metalloproteases from *X. nematophila* strain Brecon[22], and *F. psychrophilum*[48].

The effect of metal ions on the activity of *X. nematophila* PAII revealed that Zn²⁺, Ba²⁺, Mg²⁺, Ca²⁺, Li⁺ and Mn²⁺ had only a slight or no inhibitory effect, whereas Ca²⁺, Hg²⁺, and Cu²⁺ ions exhibited strong inhibitory effect ranged from 89.6 to 91.7% on the enzyme activity (Table 3). Similarly, alkaline

**Table 3:** Effect of metal cations on the activity of *X. nematophila* PAII

<table>
<thead>
<tr>
<th>Metal*</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
<td>0.0</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>10</td>
<td>3.7</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>10</td>
<td>10.6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10</td>
<td>6.9</td>
</tr>
<tr>
<td>LiCl</td>
<td>10</td>
<td>5.5</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>10</td>
<td>90.9</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>10</td>
<td>9.3</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>10</td>
<td>8.6</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>10</td>
<td>89.6</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>10</td>
<td>91.7</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>10</td>
<td>12.7</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>10</td>
<td>18.6</td>
</tr>
</tbody>
</table>

The enzyme was preincubated for 15 min at 37°C with 10 mM of the metal cations listed individually prior to substrate addition. Activity in absence of metal cations was taken as 100%. Each value represents the average of two experiments.

**Table 4:** Relative activity of *X. nematophila* PAII towards different native protein substrates.

<table>
<thead>
<tr>
<th>Protein substrate</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>100</td>
</tr>
<tr>
<td>Albumin</td>
<td>37</td>
</tr>
<tr>
<td>Gelatin</td>
<td>69</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>46</td>
</tr>
<tr>
<td>Fibrin</td>
<td>16.4</td>
</tr>
<tr>
<td>Collagen</td>
<td>4.7</td>
</tr>
</tbody>
</table>

All assays were performed in 100 mM Tris-HCl buffer, pH 8.5 at 37°C and 2 mg of each substrate. Each value represents the average of two experiments. The specificity of different microbial metalloproteases from *A. faecalis*[50] and neutral metalloproteases from animal pathogenic bacterium *V. fluvialis*[37] were inhibited by Cu²⁺ and Hg²⁺. While *Y. ruckeri* metalloprotease was enhanced by Ca²⁺ and Mg²⁺ and inhibited by Mn²⁺ and Zn²⁺[48]. From the above results we can concluded that *X. nematophila* PAII is an alkaline metalloprotease.

This class of protease, alkaline metalloprotease, is produced by several species of pathogenic bacteria, including fish pathogen *Y. ruckeri* and human pathogen *P. aeruginosa* PseA[22]. It is produced also by several species of insect pathogenic bacteria associated with the entomopathogenic nematodes incuding *P. luminescens* W14 and *P. temperata* K122[41], *Photobacteriurn* sp. strain Az29[11] and *X. nematophila* strain Kraussei[24].

The specificity of different microbial metalloproteases towards protein substrates has been reviewed[22]. Generally, they have a broad range of peptide and protein substrates[36] (Miyoshi and. In the present study, the substrate specificity of *X. nematophila* PAII indicated that it was active on a variety of natural protein substrates (Table 4). The enzyme exhibited the highest activity towards casein and albumin, but hardly hydrolyse collagen. This results are in agreement with those reported for microbial metalloproteases from *P. aeruginosa* PseA[22], *A. faecalis*[50], *P. temperata* K122[44,53] and *F. psychrophilum*[48]. The undetectable cleavage of collagen by *X. nematophila* PAII herein was in agreement with that recorded for alkaline and neutral metalloproteases from *P. luminescens* strain W14[6] and *X. nematophila* strain Brecon[22], respectively. The inability of *X. nematophila* PAII to degrade certain substrate, collagen, suggests a degree of specificity that might be physiologically important.

Bacterial pathogens produce various proteases, which contribute to the microbial virulence by hydrolyzing biologically important proteins and peptides[39,36]. However, we have no indication of the
function of X. nematophila PAI in vivo. The broad substrate specificity of the enzyme towards different proteins referred to the ability of X. nematophila PAII to digest some proteins of the insect hemolymph as reported by Bowen et al.,[8] and Cabral et al.,[11]. The hydrolysis of these proteins may provide nutritional factors to the associated nematode necessary for its complete development and reproduction inside the infected insect cadaver.[12,13,5,11] Other possible function of metalloprotease for X. nematophila PAII including the inactivation of non-specific insect’s defence mechanism could be postulated as reported by Caldas et al.,[12] and Marokhazí et al.,[12].

Despite the broad substrate specificity of X. nematophila PAII, 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 inability of X. nematophila PAII to degrade collagen, the primary component of the nematode’s cuticle,[11], 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.

In summary, purification and characterization of the predominant protease isoenzyme secreted by X. nematophila BA2 was described. X. nematophila PAII is an alkaline metalloprotease. Its substrate specificity strengthens the possibility that it is involved in degradation of insect tissues for providing nutrients to the associated nematode, which is unable to grow on insects without a previous bioconversion of the insect cadavers by the symbiotic bacteria.

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REFERENCES


