Proteolytic compartmentalization and activity in the midgut of *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae)

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Abstract

Digestive proteolytic activity in the alimentary canal of *Andrallus spinidens*, a potential biocontrol agent of lepidopteran larvae, was studied by considering enzyme compartmentalization and diversity. The alimentary canal of adults consists of a foregut, a four- sectioned midgut, namely V_1 to V_4 (ventriculus), and a hindgut. The optimal pH for general proteolytic activity was found to be at pH 8 with a small peak at pH 6. Results revealed that there are several specific proteases in the midgut of *A. spinidens,* including trypsin-like, chymotrypsin-like, and elastase as serine proteases, and cathepsins B, L and D as cysteine proteases, in addition to two exopeptidases of carboxy- and aminopetidases. Compartmentalization of digestive proteolytic activity showed that V_3 is the main area of proteolytic secretion for both general and specific proteases and that V_4 has the lowest enzymatic role, so that four out of the eight specific proteases found showed no activity in this section. The lowest and the highest proteolytic activity was found to be in the 1st and 4th nymphal instars, respectively. Using the specific inhibitors phenylmethylsulfonyl fluoride, Na-p-tosyl-L-lysine chloromethyl ketone, N-tosyl-L-phenylalanine chloromethyl ketone, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, cystatin, phenanthroline and ethylendiamidetetraacetic acid, we verified the presence of all specific proteases noted using both biochemical assays and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Our findings demonstrated that *A. spinidens* could utilize several caterpillars because of the presence of various of proteases in its midgut.

Introduction

Intensive spraying of rice fields in northern Iran with diazinon, chlorpyrifos, padan and fipronyl has caused acute and chronic effects on human and other non-target organisms as well as pest resistance. Although an egg parasitoid, *Trichogramma* spp., has annually been used to suppress population outbreaks of *Chilo suppressalis* Walker (Lepidoptera: Crambidae), economic damage still occurs. Another potential biocontrol agent is *Andrallus spinidens* F. (Hemiptera: Pentatomidae), a predator of larvae, which has been given less consideration. *A. spinidens* is widely distributed around the world (Nageswara Rao, 1965) and is a specialist on rice pests in India, Malaysia and Iran (Nageswara Rao, 1965; Manley, 1982; Mohaghegh & Najafi, 2003). Both nymphs and adults are noted to feed on several caterpillars like *Chilo suppressalis* Walker (Lepidoptera: Crambidae), *Naranga aenescens* Moore (Lepidoptera: Noctuidae) and *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) in rice fields of northern Iran (Mohaghegh & Najafi, 2003). *A. spinidens* has five generations per year, lives for 65 days and hibernates as an adult among rice debris and weeds (Zibaee *et al.*, 2012).

A. spinidens, like other hemipteran predators, uses extra-oral digestion by injecting digestive enzymes into prey to obtain nutrients required for growth and reproduction (Cohen, 1998; Zibaee *et al.*, 2012). These predators pump in potent hydrolytic salivary secretions and suck in the liquefied prey parts, delivering nutrients to their digestive tract (Cohen, 1993, 1995). Advantages of this feeding strategy include: i) the ability to circumvent the cuticle as a feeding barrier that restricts assess to nutrients; ii) the ability to bypass defensive chemicals that cause their prey to be unpalatable; and iii) the ability to allow the use of larger prey by relatively small predators (Cohen, 1998). In this case, digestive proteases have critical roles in both the digestion of proteins in the bodies of prey to serve as a main source of nutrients, and detoxification of potential defensive chemicals of animal or plant origin. A previous study revealed that salivary glands of *A. spinidens* have two anterior, two lateral and two posterior lobes (Zibaee *et al.*, 2012). General proteolytic activity in the salivary glands demonstrated an optimal pH of 8 and an optimal temperature

of 40°C when azocasein was used as a substrate. By using specific substrates, we found that trypsin-like, chymotrypsin-like, aminopeptidase and carboxypeptidase are active proteases in the salivary glands of *A. spinidens* by maximal activity of trypsin-like proteases in addition to their optimal pH of 8-9 (Zibaee *et al.*, 2012). Use of specific inhibitors including soybean trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), Na-p-tosyl-L-lysine chloromethyl ketone (TLCK), and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), significantly decreased enzymatic activity in both assessment of enzymatic condition and through the use of polyacrylamide gel electrophoresis as well as assays phenanthroline, ethylene glycol tetraacetic acid and triethylenetetramine hexaacetic acid as specific inhibitors of metalloproteases (Zibaee *et al.*, 2012). Endogenous trypsin inhibitors extracted from *Chilo suppressalis*, *Naranga aenescens*, *Pieris brassicae*, *Hyphantria cunea* and *Ephestia kuhniella* had different effects on the trypsin-like protease activity of salivary glands so that the endogenous inhibitors significantly decreased the enzyme activity except for *C. suppressalis* (Zibaee *et al.*, 2012).

Taking into account the results of our previous study and regarding the importance of *A. spinidens* as a potential biocontrol agent, we felt it was necessary to investigate the feeding strategy and physiology as well as the ecology of the insect. Therefore, the aim of the current study was to characterize the digestive proteases in the midgut of *A. spinidens* by using specific substrates and diagnostic inhibitors. A major objective of sustainable agriculture is to ensure the compatibility of pest control procedures with beneficial predator and parasitoid species in order to achieve enhanced control. The information obtained here could allow us to predict the outcome when this economically important beneficial insect feeds on phytophagous pests. For instance, whether it would be ecologically compatible, or else detrimental, as through the secondary ingestion of plant materials in the pest species' body that have been modified to express protease inhibitors.

Materials and methods

A. spinidens rearing

A colony of *A. spinidens* was established from adults collected from harvested rice fields in Amol (Mazandaran, North of Iran), in late September 2011. Insects were reared on the fifth instars of *C. suppressalis* L. (Lepidoptera: Crambidae) as a food source and were provided with wet cotton plugs fitted into small plastic dishes (2.5 cm diameter) to serve as moisture sources, and held at 25±1°C and 80% relative humidity.

Sample preparation

Soluble fractions

Insects were randomly selected and the midguts (except for 1st and 2nd instar nymphs for which the whole body was used in the experiments) were removed by dissection under a stereomicroscope in an iced saline buffer (NaCl, 10 mM). The bodies were cut with a scalpel to reveal the midgut, which was accessible after removal of fat bodies and other undesirable organs. The midgut was separated from the insect body, rinsed in iced distilled water, placed in a pre-cooled homogenizer and ground up before centrifugation. Equal portions of midgut and distilled water were used to obtain the desired concentration of the enzymes (W/V). Homogenates were transferred separately to 1.5-mL centrifuge tubes and centrifuged (Kokusan Enshinki Co. Ltd., Tokyo, Japan) at 13,000 rpm for 15 min at 4°C. The supernatants were pooled and stored at –20°C for subsequent analyses (Zibaee *et al.*, 2012).

Microvillar-bound fraction

For solubilisation of microvillar-bound enzymes (exopeptidases) in Triton X-100, membrane preparations (from the primary centrifuge precipitates, above) were exposed to Triton X-100 for 20 h at 40°C, at a ratio of 10 mg of Triton X-100 per mg of protein, before being centrifuged at 13,000 rpm for 30 min. No sediment was visible after centrifuging this supernatant at 10,000 rpm for 60 min. The activity of the enzymes remains unchanged at –20°C, for periods of at least a month, according to previous studies (Ferreira & Terra, 1983; Zibaee, 2012).

General proteolytic assay

Azocasein 2% (Sigma Aldrich Co., St. Louis, MO, USA) in universal buffer (see below: 2 mM, containing succinate, glycine and 2-morpholinoethanesulfonic acid; pH range 3-12) (Frugoni, 1957) was used to determine general proteolytic activity in the midgut of *A. spinidens* adults, based on a method described by Elpidina *et al.* (2001). The reaction mixture consisted of 50 μL of appropriate buffer solutions, 20 μL of azocasein and 20 μL of enzyme solution. After incubation at 37°C for 60 min, proteolysis was stopped by the addition of 100 μL of 30% trichloroacetic acid (TCA). Precipitation was achieved by cooling at 4°C for 10 min, and then centrifuging at 13,000 rpm for 10 min. An equal volume of 2 M NaOH was added to the supernatant and the absorbance was recorded at 450 nm (Awareness Inc., Burlington, MA, USA). A blank solution consisting of all the above components except for the enzyme solution served as a control.

Optimal pH and temperature for general proteolytic activity

The reaction mixture was the same as described above, but a different pH range of universal buffer (3-12) and temperature (20-70°C) were considered, to find the optimal pH and temperature. For the optimal pH assays, 40 μL of buffer solution (at different pH levels) was incubated with azocasein as a substrate for 10 min at 30°C as a standard temperature. Then 20 μL of sample were added and the experiment was repeated as above. For the optimal temperature assays, 40 μL of buffer solution (at the optimal pH determined) was incubated with azocasein as a substrate for 10 min at different temperature regimes from 20-70°C. After addition of 20 μL of the sample, the reaction mixture was incubated at each given temperature for 60 min. The remaining steps were repeated as above.

Specific protease assays

Serine proteases

Trypsin-like, chymotrypsin-like, and elastase-like activities (as the three subclasses of serine proteases) were assayed using a 1-mM concentration of nabenzoyl-L-arginine-p-nitroanilide (BApNA), 1 mM of N-succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (SAAPPpNA), and 1 mM N-succinyl-alanine-alanine- alanine-p-nitroanilide (SAAApNA) (all from Sigma Aldrich) as substrates, respectively. The reaction mixture consisted of 35 μL of universal buffer (pH 8 as the recommended pH for serines), 5 μL of each of the substrates and 5 μL of enzyme solution. The reaction mixture was incubated at 30°C for a period of 0-10 min before adding 30% TCA to terminate the reaction. The absorbance of the resulting mixture was then measured spectrophotometrically at 405 nm by *p*-nitroaniline release. To prove the specific proteolytic activity, a negative control was provided separately for each substrate that contained all above components except for the enzyme pre-boiled at 100°C for 30 min (Oppert *et al.*, 1997).

Cysteine proteases

Cathepsin B, L and D activities (as the three subclasses of cysteine proteases) were assayed using a 1-mM concentration of Z-Ala-Arg-Arg 4-

metjoxy-β-naphtylamide acetate, N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (both from Sigma Aldrich) and cathepsin D (Sigma-Aldrich) as substrates, respectively. The reaction mixture consisted of 35 μL of universal buffer (pH 5 as the recommended pH for cysteines), 5 μL of each substrate and 5 μL of enzyme solution. The reaction mixture was incubated at 30°C for a period of 0-10 min before adding 30% TCA to terminate the reaction and read at 405 nm. To prove the specific proteolytic activity, a negative control was provided separately for each substrate that contained all the above components except for the enzyme pre-boiled at 100°C for 30 min (Oppert *et al.*, 1997).

Exopeptidases

Activities of the two exopeptidases in the midgut of *A. spinidens* were obtained by using hippuryl-L-arginine and hippuryl-L-phenilalanine (both from Sigma Aldrich) for carboxy- and aminopeptidases, respectively. The reaction mixture consisted of 35 μL of universal buffer (pH 7 as the recommended pH for exopeptidases), 5 μL of each substrate and 5 μL of enzyme solution. The reaction mixture was incubated at 30°C for a period of 0-10 min before adding 30% TCA to terminate the reaction and read at 340 nm. To prove the specific proteolytic activity, a negative control was provided separately for each substrate that contained all the above components except for the enzyme pre-boiled at 100°C for 30 min (Oppert *et al*., 1997).

Protease compartmentalization in the four sections of midgut

To determine proteolytic compartmentalization, four-sectioned midguts of *A. spinidens* were separated and protease activities were measured as described above. V_1 to V_4 (ventriculus) sections of 10 adults were dissected and pooled separately with an equal volume of distilled water to obtain the enzymatic source.

Proteolytic profile in different nymphal instars

General and specific proteolytic activities were measured in the five nymphal instars of *A. spinidens* to find their differences depending on developmental stage. Whole-body homogenates for total proteolytic activity were extracted for 1th and 2nd instars, and from only the midgut for the other stages. The procedure was conducted as above (Oppert *et al.*, 1997).

Optimal pH determination of specific proteases

Universal buffer was used to find the optimal pH of the serine and cysteine proteases as well as the exopeptidases by using the specific substrates described. The reaction mixture and experiment procedure were the same as above.

Effect of specific inhibitors on proteolytic activity

The following compounds (Sigma Aldrich) were used to reveal any alteration of the proteolytic activity in the midgut of *A. spinidens*. PMSF (1, 3, 5 mM); trypsin inhibitor, TLCK (1, 3, 5 mM); chymotrypsin inhibitor, TPCK (1, 3, 5 mM); cysteine protease inhibitor, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane, 1, 3, 5 mM (E-64); cystatin (1, 3, 5 mM), and metalloprotease inhibitors, including phenanthroline and ethylendiamidetetraacetic acid (EDTA).

Electrophoresis zymogram

Electrophoretic detection (Laemmli, 1970) of proteolytic enzymes was performed using resolving and stacking polyacrylamide gels of 10% and 4%, respectively according to the method described by Garcia-Carreno *et al.* (1993) (Cleaver Scientific Swift Valley, UK) with slight modifications. Non-reducing polyacrylamide gel electrophoresis (PAGE) was carried out at 4°C at a constant voltage of 75 mV when the dye reached the bottom of the glass, and the gel was carefully separated and put into universal buffer for 15 min. Gels were then washed in water and put in a solution containing casein (1%) and universal buffer (pH 8). The gel was fixed and stained overnight with 0.1% Coomassie brilliant blue R-250 in methanol:acetic acid:water (50:10:40). Destaining was carried out in methanol:acetic acid:water (50:10:40) for at least 2 h. Characterization of protease classes in sodium dodecyl sulphate-PAGE zymograms using specific inhibitors was done according to Garcia-Carreno *et al.* (1993) with some modifications. A total of 25 μL of the enzyme extract was mixed with 15 μL of inhibitors at a 5-mM concentration (preparation of this sample was made 15 min before onset of the experiment.), including PMSF, TLCK, TPCK, E-64, cystatin, phenanthroline and EDTA for 30 min prior to loading. Electrophoresis and zymogram were carried out as described above.

Protein determination

Protein concentration was measured according to the method of Lowry *et al.* (1951).

Statistical analysis

The experimental design was based on completely randomized statistics and Tukey's test was used to compare data. Statistical differences were considered at P≤0.05 (SAS, 1997).

Table 1. Proteolytic compartmentalization in four sections of *A. spinidens* midgut.

Proteases	V_1	V_2	V_3	V_4
Total	0.036±0.0005[c]	0.05±0.012[b]	0.411±0.06[a]	0.058±0.005[b]
Trypsin-like	0.48±0.028[c]	20.00±1.89[b]	46.32±11.98[a]	-
Chymotrypsin-like	9.72±0.83[b]	-	43.96±9.37[a]	-
Elastase	2.75±0.27[b]	0.37±0.06[c]	3.55±0.55[a]	2.40±0.92[b]
Cathepsin B	53.79±3.53[a]	2.22±0.35[b]	0.61±0.003[c]	-
Cathepsin L	72.43±2.78[a]	64.07±11.02[ab]	54.78±5.47[b]	54.55±0.54[b]
Cathepsin D[3]	2.91±0.26[a]	-	1.86.86±0.072[b]	1.38±0.074[b]
Aminopeptidase	11.18±0.87[a]	5.74±0.99[b]	1.01±0.11[c]	-
Carboxypeptidase	34.67±2.56[b]	76.85±5.71[a]	81.15±12.98[a]	12.86±0.33[c]

V stands for ventriculus and numbers refer to section of the midgut by ordering. [a,b,c] Different letters show statistical differences in each row. Total protease and Cathspin D activities has been shown as absorbance at 410 nm.
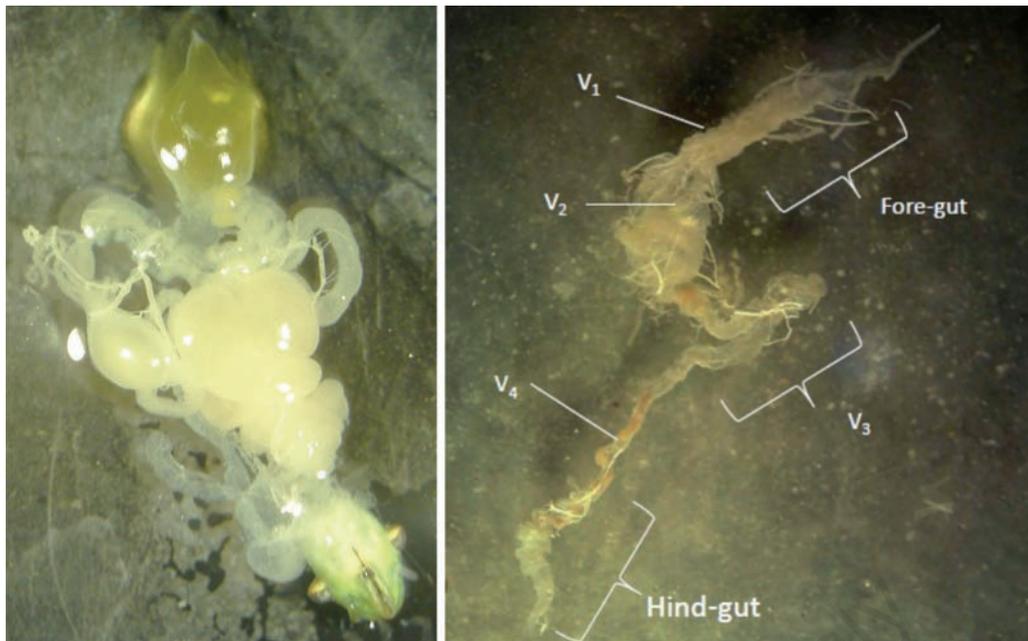
Figure 1. Alimentary tract morphology of *A. spinidens*. Image shows a complex consisting of three sections: foregut, midgut and hindgut; midgut has been divided into V_1 to V_4.

Results

Dissection of adults under a stereomicroscope revealed that the alimentary canal of *A. spinidens* consists of a foregut, a 4-sectioned midgut (V_1-V_4) and a hindgut (Figure 1). In the intact alimentary canal, the region from V_2 to the hindgut occurs as a complex so that these sections are not observable separately (Figure 1, top).

Soluble fractions prepared from the midgut of *A. spinidens* showed proteolytic activity to be dependent on pH and temperature. Specifically, activity peaked at pH 6, but a statistically different optimal pH was observed at pH 8 (Figure 2). An optimal temperature of 25°C was observed, above which proteolytic activity sharply decreased so that no enzymatic activity was observed at temperatures of 60 and 70°C (Figure 2).

Compartmentalization of proteolytic activity in different parts of the midgut showed the highest enzymatic activity in V_3 for both general and specific proteolytic activity (Table 1). In the case of the two exopeptidases assayed, carboxypeptidase had higher activity than aminopeptidase in all four sections of the midgut (Table 1). It was found that all three assayed cathepsins had the highest activity in V_1, with cathepsin L being the major cathespin in all sections (Table 1). Trypsin- and chymotrypsin-like serine proteases showed higher enzymatic activity than elastase in V_3 (Table 1).

Table 2 shows the differences in specific proteolytic activities in the various nymphal instars of *A. spinidens*. All assayed proteases had the lowest activity in the 1st instar and the highest was observed in the 5th instar, indicating an increase with nymphal development for the majority of enzymes (Table 2). In the 1st instars, trypsin-like proteases and cathepsin L had the highest activity, but tryspin-like proteases and cathepsin B showed the highest activities in the fifth instar (Table 2). In the case of exopeptidases, the 3rd and 5th instars showed the highest activities of the amino- and carboxypeptidases, respectively (Table 2).

Figures 3-5 show the optimal pH of specific proteases using universal buffer and specific substrates. Optimal pH of the three serine pro-
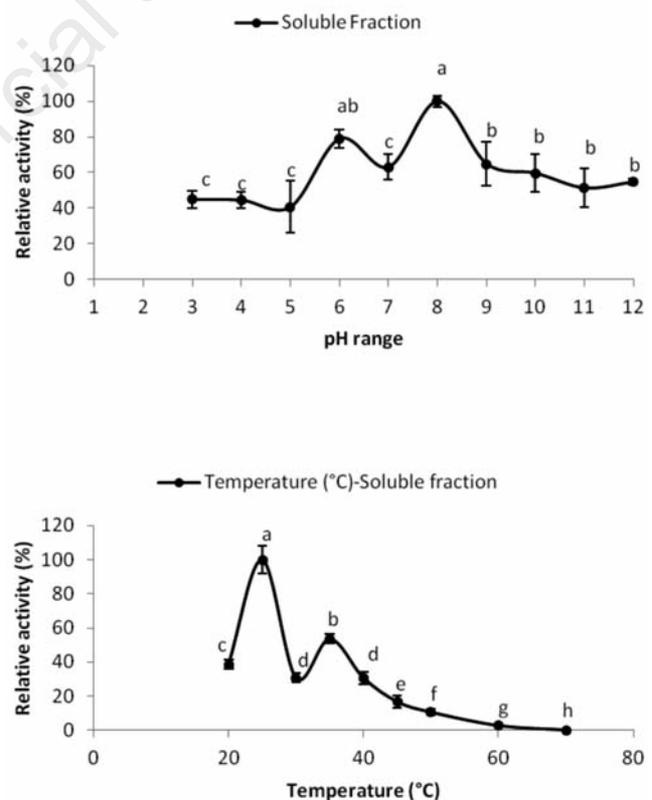
Figure 2. Optimal pH and temperature determination of general proteolytic activity in the midgut of *A. spiniden*s adults by using soluble fraction in the presence of azocasein as substrate. All values were compared by one-way analysis of variance by using Tukey's test (P≤0.05). Points marked by different letters are significantly different.

teases was found to be 9, 9-10 and 11 for tryspin-like, chymotrypsin-like and elsastase proteases, respectively (Figure 3). Optimal pH for cysteine proteases was found to be 4 for cathepsin B and 6 for both cathepsin L and D (Figure 4). Aminopeptidase had significant activity from pH 8-11, with a statistically optimal pH of 10, but carboxypeptidase showed the highest activity at pH 8 (Figure 5).

General proteolytic activity in the midgut of *A. spinidens* adults was further characterized using specific protease inhibitors (Table 3). Significant inhibition of azocaseinolytic activity by PMSF, TLCK and TPCK suggested that the serine proteases made the greatest contribution to protein digestion in the midgut (Table 3). However, considerable inhibition of azocaseinolytic activity by TLCK and TPCK showed equal importance of both trypsin-like and chymotryosin-like proteases. E-64 and cystatin also decreased proteolytic activity, but the inhibitory effect of cystatin was higher than E-64 (Table 3). Inhibition of general proteolytic activity by phenanthroline and especially EDTA revealed the presence of metalloproteases in the midgut extract of *A. spinidens* adults (Table 3).

Zymogram analysis of the compartmentalization of proteolytic activity in V_1 to V_4 of the midgut confirmed our biochemical assays. Figure 6 reveals the sharper bands of V_3 in comparison with the other sections, especially V_4, where proteolytic activity is represented by just a thin band. Zymogram analysis of proteolytic activity in the nymphal instars also confirmed the biochemical assays (Figure 7). In addition, zymogram characterization of the protease activity in the midgut of *A. spinidens* using inhibitors is shown in Figure 8. Six bands with proteolytic activity (P_1-P_6) were observed in the control. Different classes of proteases were detected in the presence of the specific inhibitors through the disappearance or reduced intensity of the bands compared with the control. Zymograms of the general serine protease inhibitor PMSF revealed a slight inhibition of band P_3 and the complete disappearance of bands P_4 to P_6 (Figure 8). Trypsin-like serine proteases were determined in zymograms using TLCK as an irreversible inhibitor of trypsin-like serine protease as band P_4 (Figure 8). TPCK, as an irreversible inhibitor of chymotrypsin- like protease, had inhibitory effects on bands P_5 and P_6 (Figure 8). Cysteine proteases were inhibited by both E-64 and cystatin through the disappearance of bands P_2 and P_3 (Figure 8). Metalloproteinases were not found in the gel system using the inhibition by EDTA and phenanthroline through the disappearance of P_2, P_5 and P_6 for phenanthroline and P_2, P_5 and P_6 for EDTA (Figure 8).

Discussion and conclusions

Results of the current study clearly indicate that digestive proteolytic activity in the midgut of *A. spinidens* is dynamic and dependent on the interactions among the site of secretion, pH and protease class. Meanwhile, compartmentalization of enzyme secretion and digestion
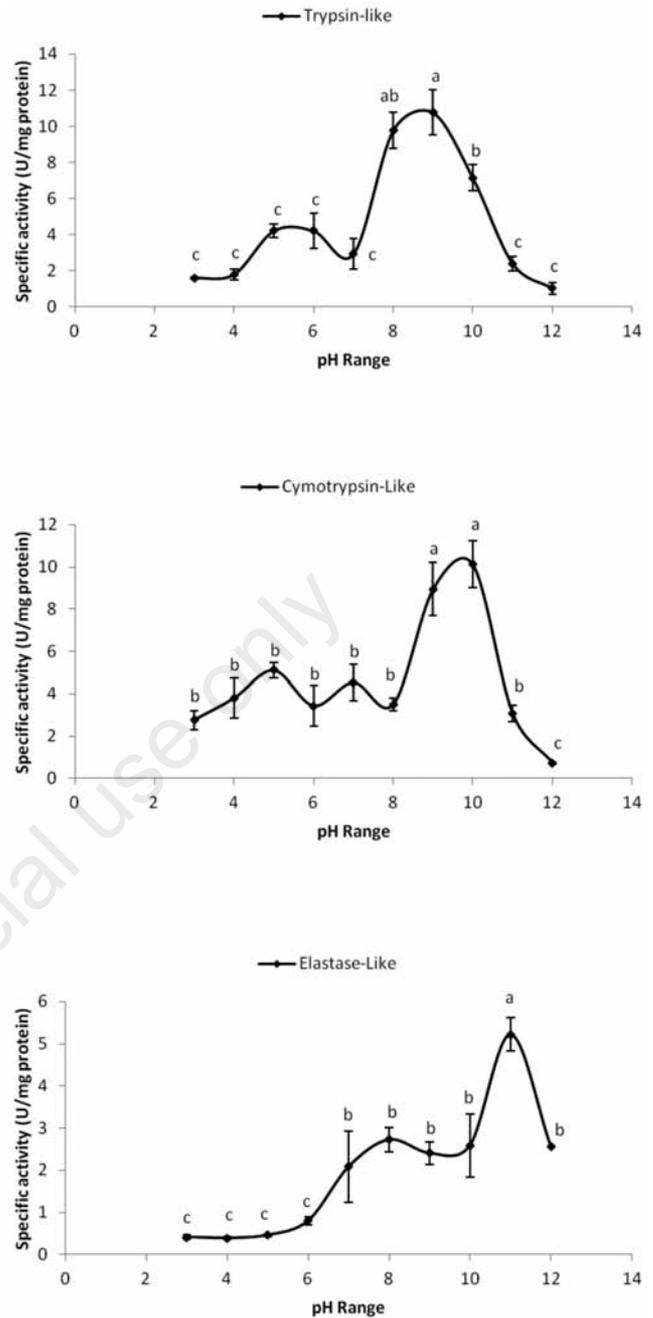
Figure 3. Optimal pH determination for serine proteases found in the midgut of *A. spinidens* adults. Values marked by different letters are significantly different (Tukey's test (P≤0.05).

Table 2. Specific proteolytic activity (U/mg protein) in the nymphal instars of *A. spinidens*.

Larval instar	Total protease	Trypsin-like	Chymotrypsin-like	Elastase	Cathepsin B	Cathepsin L	Cathepsin D	Aminopeptidase	Carboxypeptidase
1st instar	0.039±0.004[d]	6.98±0.04[c]	0[c]	2.34±0.01[c]	0[c]	10.92±0.085[bc]	0.38±0.029[b]	3.96±0.14[c]	3.29±0.85[c]
2nd instar	0.24±0.021[cd]	6.06±0.29[c]	12.45±1.65[b]	9.4±0.38[bc]	0.34±0.06[b]	20.9±0.036[b]	0.065±0.005[c]	6.63±0.098[c]	7.94±0.71[c]
3rd instar	0.33±0.017[c]	20.90±1.42[b]	11.37±3.6[b]	14.76±1.23[b]	56.13±2.19[a]	7.46±0.39[c]	0.33±0.026[b]	52.9±4.19[a]	39.76±2.12[b]
4th instar	0.51±0.017[b]	19.59±1.25[b]	42.12±3.61[a]	19.52±1.79[b]	48.42±1.82[ab]	29.12±3.25[ab]	0.58±0.093[a]	35.73±1.2[b]	54.31±5.36[a]
5th instar	0.89±0.035[a]	59.91±6.14[a]	56.34±2.25[a]	53.91±7.82[a]	100.57±7.36[a]	44.18±2.26[a]	0.57±0.025[a]	40.81±2.25[ab]	57.66±4.16[a]

Homogenates for total proteolytic activity were extracted from whole body for 1th and 2nd instars and midgut for others. The experiment was carried out at 30°C for both substrates. Total protease and Cathspin D activities has been shown as absorbance at 405 nm. [a,b,c,d] Different letters in each column show significant differences among values when Tukey's test was used at P≤0.05, n=3.

Table 3. Effect of some specific inhibitors on proteolytic activity in the midgut of *A. spinidens* adults.

Compounds	Concentration (mM)	Activity (U/mg protein)
Control	5	0.18 ± 0.06^a
PMSF	5	0.041 ± 0.003^d
TLCK	5	0.079 ± 0.008^c
TPCK	5	0.082 ± 0.002^c
E-64	5	0.133 ± 0.023^{ab}
Cystatin	5	0.073 ± 0.01^{cd}
Phenanthroline	5	0.143 ± 0.36^{ab}
EDTA	5	0.097 ± 0.008^b

Azocasein (2%) was used as substrate. ^{a,b,c,d} Statistical differences have been shown by different letters following one-way analysis using Tukey test (P≤0.05). PMSF, phenylmethylsulfonyl fluoride; TLCK, Na-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; E-64, L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane; EDTA, ethylendiamidetetraacetic acid.
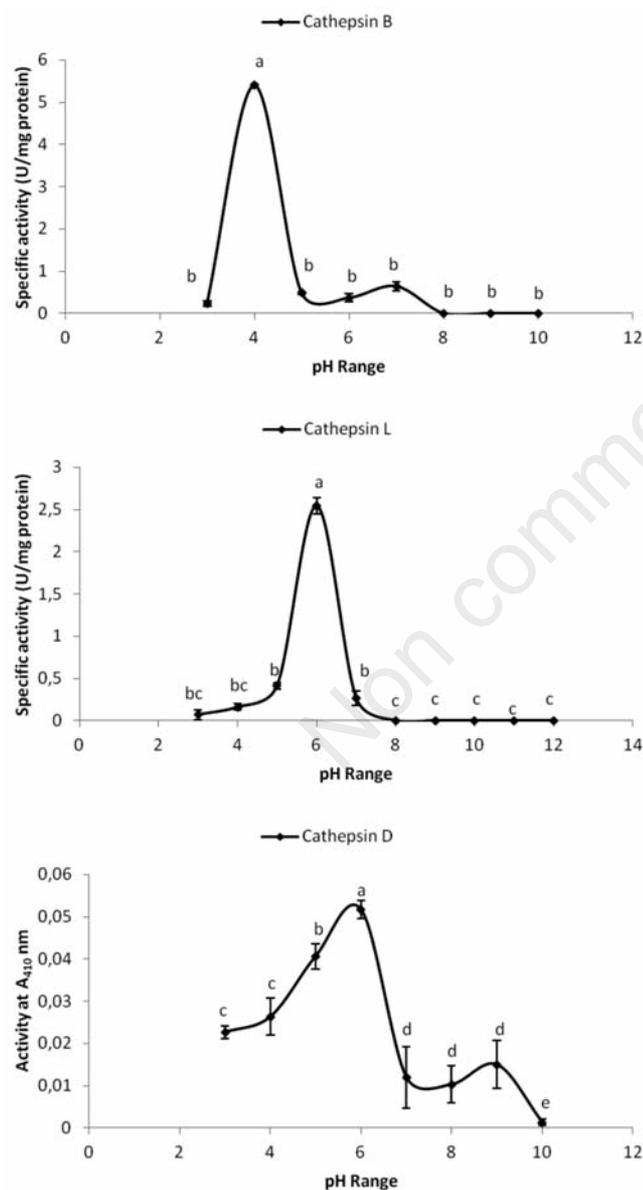
were observed when different activities of digestive proteases were obtained by biochemical assays and PAGE electrophoresis. Also, it was found that different protease classes are active in the midgut by using specific substrates and inhibitors as well as zymogram analysis.

Knowledge of the alimentary canal anatomy is necessary for a complete understanding of digestive physiology in insects. A major characteristic of the hemipteran alimentary canal is a division of the midgut into four significant sections. As Saxena (1963) described, the alimentary canal of hemipterans consists of a foregut as a short tube that contains the pharynx and esophagous. The midgut is sectioned into V_1, V_2, V_3 and V_4. V_1 commonly resembles a large sac in which some variation can be observed, depending on species. V_2 is similar to a tube and is much thinner than V_1. V_3 is a long section filled by a yellowish-brownish thick liquid. V_4 is a small tube-like section. No developed sections can be observed such as the ileum or colon in the hindgut. Our observations on the *A. spinidens* alimentary canal corresponds with the description of Saxena (1963). Briefly, the foregut is a short tube but the midgut consists of four sections, of which the first is large, and the second is thinner and smaller than the first and third. The third section is a long tube, and the fourth is a short tube similar to the hindgut. Additionally, it was found in the four sectioned midgut of *A. spinidens* that V_3 showed the highest proteolytic activity, not only for general proteases but also specific proteases, such as the trypsin-like, chymotrypsin-like, cathepsin L and carboxypeptidase. Saxena (1954) and Goodchiled (1966) have attributed these morphologies and functions to the hibernation physiology of bugs. A majority of hemipterans like *A. spinidens* hibernate as an adult, so the following conclusions could be drawn. During diapause, insects rely on food storage in the first midgut region (V_1) (Saxena, 1954), which would explain why V_1 is large and sac-like. The low amount of proteolytic activity seen in V_1 is most like-

Figure 4. pH determination for cysteine proteases found in the midgut of *A. spinidens* adults. Values marked by different letters are significantly different (Tukey's test: P≤0.05).
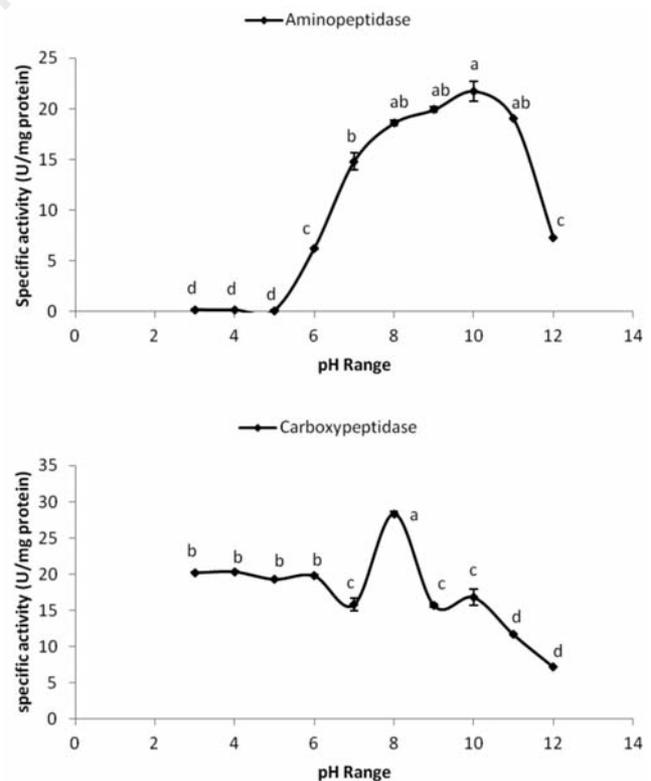
Figure 5. Optimal pH determination for two exopeptidases found in the midgut of *A. spinidens* adults. Values marked by different letters are significantly different (Tukey's test: P≤0.05).

ly due to salivary enzymes that entered the gut along with the food consumed (Saxena, 1954, 1963). The role of V_2 is to connect the main storage site (V_1) with the digestion and absorption site (V_3) (Saxena, 1954; Goodchiled, 1966). V_2 may also have some function in storage and digestion of food (Goodchiled, 1966). The fourth region of the midgut is a place where symbionts are accommodated in *Nezara viridula* L. (Hemiptera: Pentatomidae) and possibly in other hemipterans (Chapman, 1998) like *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) (Goodchiled, 1966). Additionally, it was found that the digestion process in the midgut of *A. spinidens* takes about 12-24 h, as currently seen in starved and fed adults, so the sac shape of V_1 may provide a storage chamber for the gradual digestion of food.

There are two major types of proteases in insects, known as exo- and endoproteases. Exopeptidases attack protein molecules from the N-terminal end (aminopeptidases) and the C-terminal end (carboxypeptidases) (Terra & Ferriera, 2005). The enzymes liberate one amino acid residue at each catalytic step. Endopeptidases break internal bonds of protein molecules, so there are different types of these proteases because amino acid residues vary along their peptide chain (Terra & Ferriera, 2005). There are three subclasses of endopeptidases involved in digestion according to their active site group: serine, cysteine, and aspartic proteases, and metalloproteases that are further classified as trypsin, chymotrypsin, elastase and cathepsins B, L and D (Terra & Ferriera, 2005). In serine proteases, the hydroxyl group in the side chain of a serine residue acts as a nucleophile in the reaction that hydrolyzes peptide bonds, whereas in cysteine proteases, the sulfhydryl group of a cysteine side chain performs this function (Kanost & Clem, 2011). In aspartic acid proteases and metalloproteases, a water molecule in the active site (positioned by interacting with an aspartyl group or a metal ion, respectively) attacks the peptide bond as a nucleophile factor (Kanost & Clem, 2011). The general proteolytic activity was found in the soluble fraction of *A. spinidens* adults that demonstrates the importance of site of secretion in the process of digestion. This phenomenon has received less attention by researchers. The activities of all the assayed specific proteases including serine, cysteine proteases and two exopeptidases were higher in the soluble fraction. Soluble amino- and carboxylpeptidases are found in less evolved insects (*e.g.* Orthoptera, Hemiptera, and Coleoptera: Adephaga), whereas in more evolved insects (*e.g.* Coleoptera: Polyphaga, Diptera and Lepidoptera), these enzymes are membrane-bound. For example, Zibaee (2011) found that carboxy- and aminopeptidase of *Pieris brassicae* L. (Lepidoptera: Pieridae) are mainly bound to the membranes of midgut cells. In contrast, Mehrabadi & Bandani (2011) found that both carboxy- and aminopeptidase are active in the soluble fraction and no activity was observed in the sediment fraction of the hemipteran *E. integriceps*. The majority of studies on hemipteran gut proteases have reported that the primary gut enzymes responsible for protein digestion are of the cysteine mechanistic class, with lower activity in the serine class (Terra & Ferriera, 2005). Stamopoulos *et al.* (1993) have reported only trace trypsin/chymotrypsin activity in the gut of *Podisus maculiventris* Say (Hemiptera: Pentatomidae), as have Bell *et al.* (2005) on the same insect. Against these studies, Pascual-Ruiz *et al.* (2009) showed that serine, cysteine, aminopeptidase and carboxypeptidase are the main proteases involved in protein digestion of *P. maculiventris,* so that variability of prey may affect their activity. Zhu *et al.* (2003) and Wright *et al.* (2006) have demonstrated activity of both cysteine and serine proteases in the midgut of *Lygus lineolaris* and *L. hesperus* Knight (Hemiptera: Miridae). These differences direct attention to the enzymes' site of activity and the use of a negative control, as in our study. The current study indicates the need for standardization of methods for the characterization of proteolytic activity in hemipterans and all insects. Also, serine activity in the midgut of *A. spinidens* could be attributed to the ingestion of salivary serines or utilization of prey serine proteases. Pascual-Ruiz *et al.* (2009) demonstrated that the rel-

ative activity of serine proteases in the midgut of *P. maculiventris* depends on the prey. The authors suggested that *P. maculiventris* may utilize prey proteolytic enzymes for digestion of their own tissues. Their conclusion is based on the increase of trypsin and chymotrypsin activity observed in *P. maculiventris* feeding on lepidopteran larvae in comparison with those feeding on beetles or dipteran pupae (Pascual-Ruiz *et al.*, 2009).

Optimal pH determination revealed an alkaline pH for total proteolytic activity, serine proteases and two exopeptidases with a another peak at pH 6. But an acidic pH was found for cysteine proteases. An alkaline pH is typical for activity of serine proteases (*e.g.*, the trypsin-like and chymotrypsin-like proteases in our study) and exopeptidases (*e.g.*, the amino and carboxypeptidases in our study) that are known to be active

Figure 6. Zymogram of proteolytic activity in different sections of the midgut of *A. spinidens*.

at pH between 7-12 (Terra & Ferriera, 2005). Zhu *et al.* (2003) reported optimal gut proteolytic activity of *L. lineolaris* at pH 4.25 and 8.5. Wright *et al.* (2006) found acidic midgut caseinolytic activity with an optimal pH of 4.5, which was attributed to cystein proteinases. By using specific inhibitors, these authors found an optimal pH activity of 7.5, confirming serine activity in the midgut. The frequency at which insects encounter a high-pH environment in the field is unknown, as is the identity and digestibility of the most commonly ingested protein substrates. In this case, *A. spinidens* is a specific biocontrol agent of *Chilo suppressalis*. Usually, caterpillars have an alkaline pH in their gut and hemolymph to overcome secondary metabolites of ingested plants. Also, the feeding habits of *A. spinidens* require an alkaline pH in its gut for detoxifying secondary metabolites ingested along with larval body components. Also, this may represent a co-evolutionary phenomenon, as enzymes secreted in the midgut of *A. spinidens* must show stability in the highly alkaline state of caterpillars. As the gut constitutes the majority of a caterpillar's body, this conclusion seems to be more reliable.

Class-specific inhibitors are biochemically used to identify the types of proteases involved in biological processes. However, the effect of inhibitors can also be substrate-specific. Direct measurement of class-specific proteolytic activities using combinations of inhibitors to inhibit one or all classes of proteases may aid in the accurate classification and quantification of digestive proteases (Wright *et al.,* 2006). Different specific protease inhibitors including PMSF, TLCK, TPCK, cystatin, E-64, EDTA and phenanthroline were used to confirm the type of proteases present in the midgut of *A. spinidens*. PMSF highly inhibited enzymatic activity, demonstrating a major presence of serine proteases in the midgut. TLCK (a trypsin-like inhibitor) and TPCK (a chymotrypsin-like inhibitor) decreased enzymatic activity in different portions, demonstrating the equal roles of trypsin-like and chymotrypsin-like proteases. We also found cystatin and EDTA inhibitory effects, implying roles of cysteine and metalloproteinase in protein digestion by *A. spinidens*. Zhu *et al.* (2003) studied the effect of eleven specific inhibitors on the proteolytic activity of *L. lineolaris*. Bell *et al.* (2005) reported that the serine protease inhibitor PMSF caused the most marked reduction in the rate of proteolysis of *P. maculiventris*. Proteolytic hydrolysis was inhibited by PMSF, TPCK and E-64 in *Brachynema germari* Kolenati (Hemiptera: Pentatomidae), indicating the presence of chymotryptic and cysteine activity in the midgut of this insect (Bigham & Hosseini-Naveh, 2010). Presence of serine proteases in the digestive system of hemipterans and other insects is well documented. The midgut extract from *Rhodnius prolixus* L. (Hemiptera: Reduviidae) also showed hydrolysis of a tryspin-like substrate (Houseman, 1978). In gel assays, six proteolytic bands were observed, although the partial denaturing methodology of the electrophoresis does not preclude the presence of further proteolytic enzymes that were not detected. Disappearance of proteolytic bands caused by specific inhibitors verified biochemical assays for the presence of specific inhibitors. Bell *et al*. (2005) found four proteolytic bands in *P. maculiventris* and reported inhibitory effects of E-64, evidenced by the disappearance of bands.
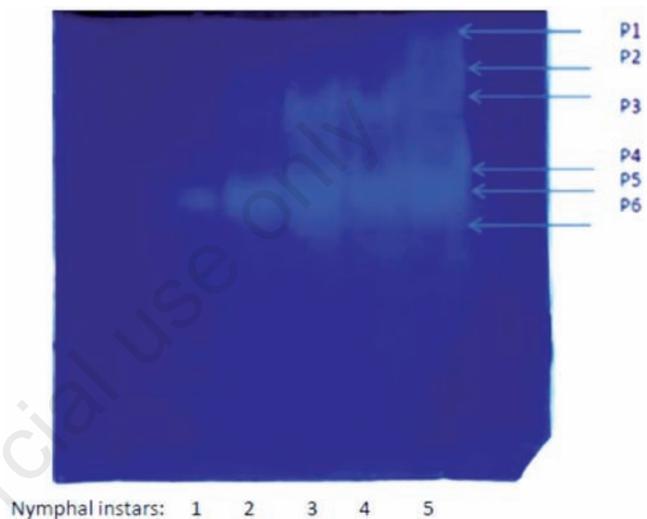
Figure 7. Proteolytic zymogram in five nymphal instars of *A. spinidens*. From left to right, 1st to 5th nymphal instars.
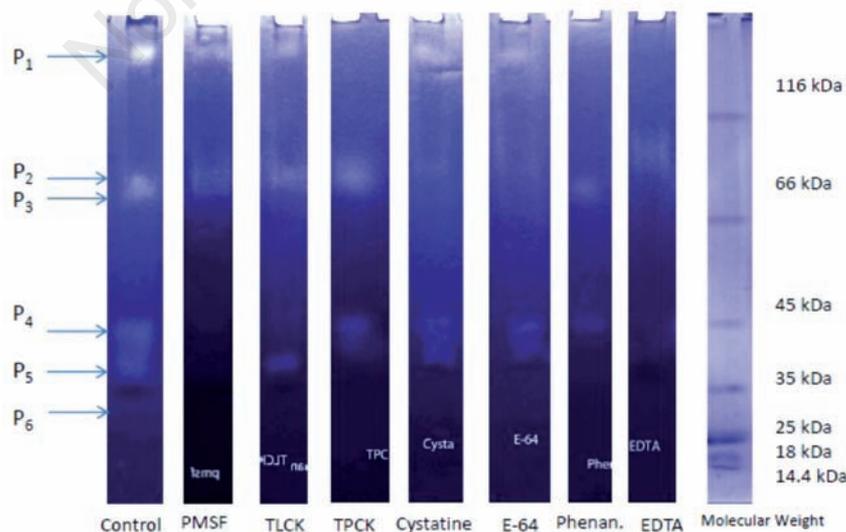
Figure 8. Zymogram analysis of the digestive proteolytic activity in *A. spinidens* to prove the presence of specific proteases by using inhibitors.

Our findings in these studies show that *A. spinidens* utilizes several endo- and exopeptidases for gut proteolysis of ingested food, as those are sensitive to inhibitors of a given mechanistic class. Due to the inhibition of proteases in *A. spinidens* by tryspin and cysteine protease inhibitors, use of these toxic proteins in transgenic rice must be considered to prevent their possible negative effects on this predatory bug. In addition, these findings will help us to purify and characterize these proteases in future work, and to determine their importance in the digestion of prey proteins in different host regimes. This research is being continued to determine the responses of *A. spinidens* digestive enzymes after feeding on different prey, elucidation of the secretion mechanism in starved and fed insects in both *in vivo* and *in vitro* conditions, as well as the extraction of endogenous inhibitors of the known digestive enzymes.

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