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Biochemical characterisation of the tissue degrading enzyme, collagenase, in the spined soldier bug, *Podisus maculiventris* (Hemiptera: Pentatomidae)

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Abstract: *Podisus maculiventris* (Say) is a generalist predator attacking many insect species from different orders. The bug injects saliva into its prey's body. The ingested hemolymph and liquefied internal tissues pass through the bug's alimentary tract. Collagenase working on peptide bonds of collagen and basement membrane proteins, leads to the disintegration of the prey's internal organs. As yet, there is an almost complete lack of knowledge on the collagenase activity in *P. maculiventris*. The collagenase activity of the salivary glands and midgut was optimum at pH 8.0 which was congruent with the optimal pH of the total proteolytic activity of the salivary glands. More collagenolytic activity was determined in the posterior lobe of the salivary glands and anterior midgut. Significant inhibition of collagenolytic activity by ethylenediaminetetraacetic acid (EDTA) revealed the enzyme is a metalloproteinase. The collagenase activity notably decreased when the bug went hungry. The salivary gland collagenase is a vital enzyme in extra-oral digestion and facilitates the action of other digestive enzymes. The midgut collagenase may be involved in the digestion of the ingested muscle fibers. The collagenase probably acts as an intoxicating agent in the saliva (venom) of *P. maculiventris*. Paralysing toxins are present in the salivary gland secretion.

Key words: collagenase, EDTA, extra-oral digestion, *Podisus maculiventris*, starvation

Introduction

Many asopin predatory stink bugs (Hemiptera: Pentatomidae: Asopinase) including *Perillus* spp., *Picromerus* spp., and *Podisus* spp. are major predators of crop pests especially lepidopteran larvae (De Clercq *et al*. 1998; De Clercq 2000). The spined soldier bug, *Podisus maculiventris* (Say), is a generalist predator known to attack more than 90 insect species from eight orders (McPherson 1980). All the nymphal stages and adults are predators, except the first nymphal stage which requires only water or plant sap for normal development (De Clercq 2002). *P. maculiventris* is endemic in North America and was introduced to Europe and other regions from North America. Today it is known as one of the most important natural enemies in greenhouses endangered by larval pests (De Clercq 2000; Van Lenteren *et al*. 2003). *P. maculiventris*, like other asopine bugs, relies on extra-oral digestion (EOD) a process in which the predator liquefies its host's tissues by injecting toxins and digestive enzymes into its body (Cohen 1995) and subsequently sucks the resultant soupy food by a powerful pharyngeal pump. Extra-oral digestion is considered to be an initial digestion while the final and main digestion occurs in the predator's midgut (Fialho *et al*. 2012). The predators are well-enabled to explorate large prey using EOD (Cohen 1995). Due to the presence of degrading enzymes in the saliva of *P. maculiventris*, and appropriate morphological and behavioral mechanisms, the performance of EOD seems to be more than 94% if the outer skeleton of the prey remains safe during feeding (Cohen 1998). The basis of digestion is to break macro-molecules into simpler absorbable compounds by digestive enzyme actions (Terra and Ferreira 1994). The process of EOD also depends completely on the presence of different digestive enzymes in the saliva. Thus, the presence of hydrolyzing enzymes such as trypsin, chymotrypsin, phospholipase, lipase, amylase, and collagenase in the saliva of these insects seems to be reasonable (Cohen 1995; Bell *et al*. 2005; Oliveira *et al*. 2006; Fialho *et al*. 2012).

In particular, collagenase acting on the collagen present in the intercellular spaces, contributes to degradation and liquefaction of the host's tissues (Fialho *et al*. 2012). Collagenase is an enzyme belonging to the matrix metalloproteinases. These proteinases use zinc as a cofactor for the activity. The effect of collagenase on peptide bonds of extra cellular matrix collagen, and basement membranes caused the degradation of prey internal tissues. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) can impair the activity of collagenases by removing zinc (Boulard and Garrone 1978). Collagenase activity in insects has been studied in some flesh flies (Boulard and Garrone 1978; Lecroisey *et al*. 1979; Bowles *et al*. 1988) and

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in *P. nigrispinus* (Fialho *et al*. 2012). In the current study, some biochemical characteristics of collagenase were revealed in the salivary glands and the midgut of *P. maculiventris*.

Materials and Methods

The insect and the rearing

All developmental stages of *P. maculiventris* were maintained on a diet of the pyralid larvae, *Galleria mellonella*, in a growth chamber under 24±1°C with a photoperiod of 16 : 8 h (light : dark cycle) and 70±5% relative humidity. The greater wax moth larvae were fed and maintained on natural wax and an artificial diet (containing wax, honey, glycerol, flour, and yeast) under the laboratory conditions of 28±2°C with a photoperiod of 16 : 8 h (light : dark cycle) and 50±5% relative humidity.

Sample preparation

Enzyme extracts of the midguts and the salivary gland complex of adults and different nymphal instars were prepared according to Aghaali *et al*. (2013) and Darvishzadeh *et al*. (2013) with slight modifications. Briefly, five-day old adults were randomly selected from the colony and were chilled for one hour on ice, to render them immobile. The midguts and the salivary gland complex were removed and the midguts were separated into anterior midgut, median midgut, and posterior midgut. The salivary glands were separated into anterior lobe, posterior lobe, and accessory gland. The separated parts of the midgut and the salivary gland complexes were rinsed in ice-cold distilled water and placed in a pre-cooled, hand-held, glass homogeniser. The homogenates were separately transferred to new 1.5 ml microtubes and centrifuged at 15,000 g for 20 min at 4°C. The resulted supernatants were pooled and stored at –20°C until used.

Proteolytic assay

General proteolytic activity of the midgut and the salivary gland extracts was determined using the substrate bovine hemoglobin at a broad pH range (pH 2 to pH 12). A universal buffer system (40 mM sodium citrate-phosphate-borate) was used to determine the optimum pH of proteolytic activity (Hosseininaveh *et al*. 2007). The assay was performed according to Cohen (1993) with slight modifications. Ten µl hemoglobin solution (1%) was added to 70 µl of the universal buffer with the desired pH. Reactions were started with the addition of 20 µl of enzyme extract at 30°C for 120 min. To stop the reaction, 100 µl of 30% trichloroacetic acid (TCA) was added to the reaction mixture. Undigested protein precipitation was achieved by cooling the TCA-treated mixture at 4°C for 45 min. Centrifugation followed at 16,000 g for 10 min. In blanks, TCA was added to the reaction mixture before the enzyme extract addition. The peptides liberated from the hemoglobin digestion were estimated by the Folin-Ciocalteu reagent (Folin and Ciocalteu 1927) at 630 nm using a microplate reader (BioTek ELx808).

Collagenase activity

Collagenase activity was determined according to Fialho *et al*. (2012) with some modifications. The activity was determined by following the release of the amino acids from bovine Achilles tendon collagen (Sigma® C9879) as substrate in 50 mM of Tris-HCl buffer pH 7.8. Briefly, 1 mg of collagen was added to 50 µl of the Tris-HCl buffer containing 1 mM $CaCl_2$ and 1 µl of the enzyme source. After 480 min at 30°C, the assay tubes were removed and were placed on ice, and 50 µl of EDTA (5 mM) and $SnCl_2$ (7 mM) were added. The tubes were then processed according to Rosen (1957) to determine free amino acids; in brief, 200 µl of citrate buffer were pipetted to each tube followed by the addition of 100 µl of ninhydrin solution. After boiling the tubes for 10 min, 1 ml of isopropanol-water (1 : 1) solution was added to each tube. After centrifugation at 16,100 g for 10 min at 4°C, the absorbance was recorded at 570 nm. The effect of pH on the collagenase activity was measured using a universal buffer (40 mM sodium citrate-phosphate-borate) at a broad pH range (pH 3 to pH 11).

Inhibition studies

The enzyme extract was pretreated with an aqueous solution in 1 and 5 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and EDTA. Incubations were conducted at the previously established pH optima at 30°C for 30 min. All incubations were conducted in triplicate alongside the appropriate controls. The results were expressed as residual activity relative to the control. In *in vivo* assays, 4 µl of the EDTA solution was injected into the larval body of *G. mellonella* at a concentration of 5 mM, in five replicates. Distilled water was injected as the control. The starved bugs were fed with the inhibitor-treated larvae and the control larvae. After 7 h, the bugs were dissected and the midgut collagenase activity was measured.

Electrophoretic zymogram

Electrophoretic detection of proteolytic enzymes was performed using resolving and stacking polyacrylamide gels of 10%T/0.87%C and 4%T/0.35%C, respectively, according to the method described by Garcıa-Carreno *et al*. (1993) with some modifications. Native-polyacrylamide gel electrophoresis (PAGE) was carried out at 4°C, and the gels were then immersed in 0.5% casein in 40 mM Tris-HCl (pH 8) for 30 min at 4°C to allow the substrate to penetrate into the gel. Then, the gels were transferred to the same solution at 25°C for 120 min. Finally, the gels were washed in water and immediately fixed and stained with 0.1% comassie brilliant blue R-250 in a solution of methanol-acetic acid-water (50 : 10 : 40). Characterisation of protease classes in native-PAGE zymogram by specific inhibitors was then performed. A total of 20 µl of the midgut extract was mixed with 20 µl of EDTA and AEBSF stock solution before electrophoresis.

Effect of starvation on collagenase activity

The fed adults went hungry for 0, 8, 16, 24, 30, and 40 h, and were fed on the larval *G. mellonella* after 40 h. The adults were dissected during each of the time intervals, and the collagenase activity was measured according to the previous sections.

Protein assay

Protein concentration was determined using the method of Lowry *et al*. (1951) with bovine serum albumin as the standard.

Results

Proteolytic assay

The pH dependence of the proteolytic activity of the salivary gland and the midgut extract from *P. maculiventris* are shown in figures 1 and 2. The results showed that the salivary glands extract hydrolysed the substrate over a broad range of acidic and alkaline (pH 2 to pH 12) with the maximum activity at pH 8. The highest proteolytic activity of anterior, median, and posterior midgut occurred at pH 4, 4, and 5, respectively.

Collagenase activity

Collagenolytic activity of the salivary gland complex, different parts of the midgut, and different nymphal instars are shown on table 1. The salivary gland collagenase activity was optimum at pH 8.0 against the substrate collagen and the activity increased in the upper incubation time (Fig. 3). Higher collagenolytic activity was determined in the posterior lobe of the salivary glands and no activity was observed in the accessory glands. The highest midgut collagenase activity was detected in the anterior section. Older nymphal instars showed more collagenase activity than those of the younger ones. However, no collagenolytic activity was observed in the first nymphal instar.

Enzyme inhibition assays

Collagenase activity of the salivary gland extract was further characterised using specific proteinase inhibitors (Fig. 4). Collagenase activity was significantly inhibited by EDTA suggesting that collagenolytic activity in the digestive system of *P. maculiventris* probably belongs to the class methalloproteinases. The inhibitor AEBSF, a specific serine proteinase inhibitor, at low (1 mM) and high (5 mM) concentrations, showed less inhibition. *In vivo* assays with EDTA, revealed a 38% inhibition of collagenase activity.

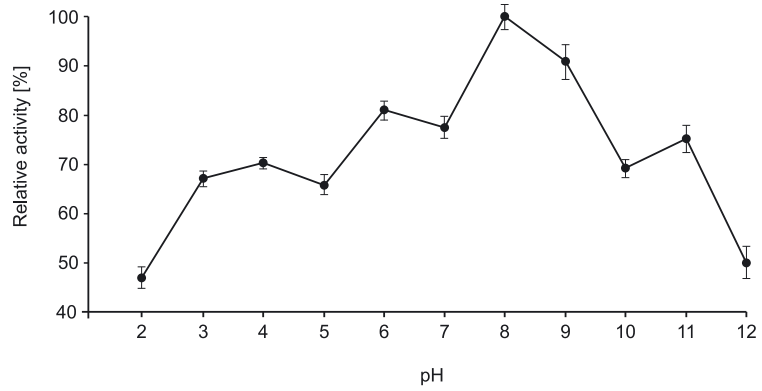

Fig. 1. The effect of pH on the proteolytic activity of salivary gland extract from *P. maculiventris* using the substrate hemoglobin. Data are means of triplicate measurements from the same pool of midgut extract with standard error of the mean

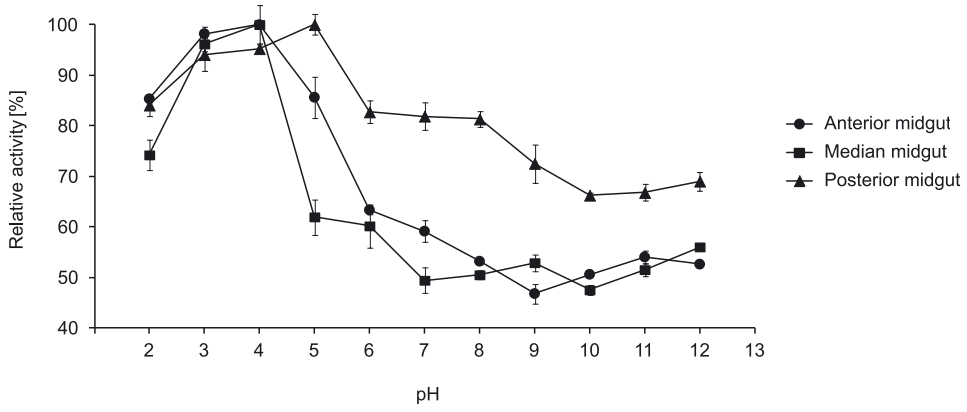

Fig. 2. The effect of pH on the proteolytic activity of midgut extract from *P. maculiventris* using the substrate hemoglobin. Data are means of triplicate measurements from the same pool of midgut extract with standard error of the mean

Table 1. Collagenolytic activity (mean ±SE) in *P. maculiventris*

Stage	Tissue	Section	Total activity* [mU]	Specific activity [mu/mg]
Adult	midgut	total	980±20	130±10
		anterior midgut	1150±60	140±10
		median midgut	730±30	90±7
		posterior midgut	370±20	40±10
	Salivary gland	total	940±90	180±15
		anterior lobe	260±90	40±7
		posterior lobe	1120±90	190±9
		accessory gland	0	0
Nymph 1	midgut	total	0	0
Nymph 2	midgut	total	400±60	90±10
Nymph 3	midgut	total	520±30	120±6
Nymph 4	midgut	total	680±70	130±4
Nymph 5	midgut	total	830±120	150±11

*one unit liberates peptides from collagen equivalent in ninhydrin color to 1.0 μmol of leucine in 480 min at pH 7.8 at 30°C in the presence of calcium ions

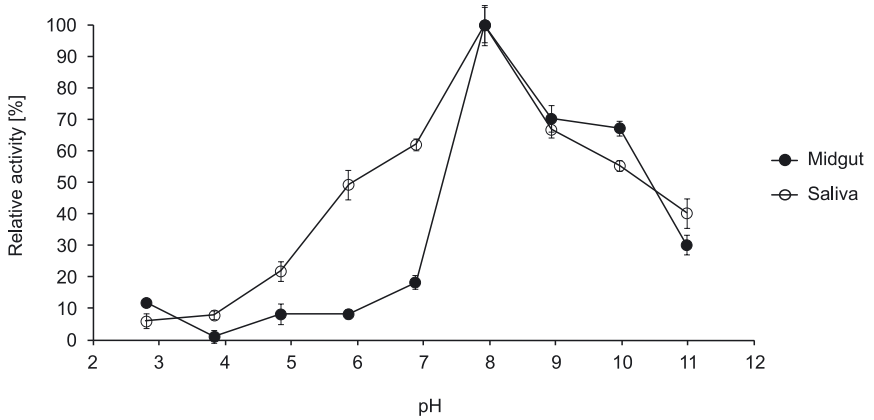

Fig. 3. Effect of pH on collagenase activity from the midgut and salivary gland of *P. maculiventris* against the substrate collagen. Each point represents the means of triplicate measurements, and the vertical bars are the standard error of the means

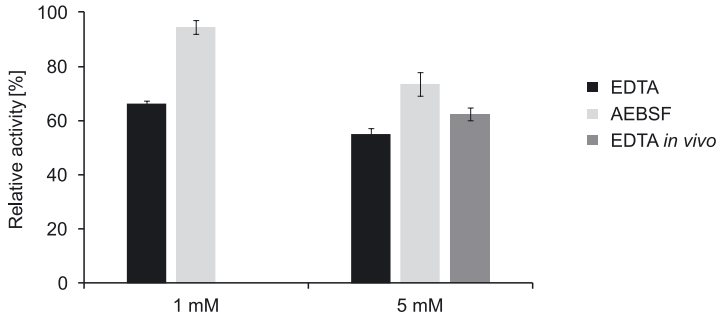

Fig. 4. *In vitro* and *in vivo* effect of the inhibitors EDTA and AEBSF on collagenase activity from the salivary gland of *P. maculiventris*

Zymogram analyses

Further characterisation of the proteinase activity of the midgut extracts from *P. maculiventris* using casein incorporated in PAGE, is shown in figure 6. At least five proteolytic bands were detected in zymogram analyses (Fig. 5). The different classes of proteases were further detected in the presence of the specific inhibitors by the disappearance or reduced intensity of the bands, compared to the control. The metalloproteinase inhibitor, EDTA, caused band 1 to completely disappeared, suggesting that the activity was inhibited with the lowest electrophoretic mobility.

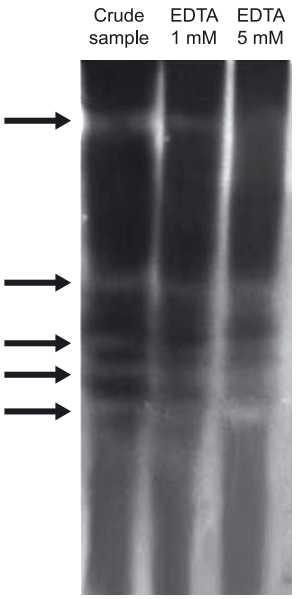

Fig. 5. Native-PAGE of proteinase activity and effect of EDTA on the Salivary gland extracts from *P. maculiventris*

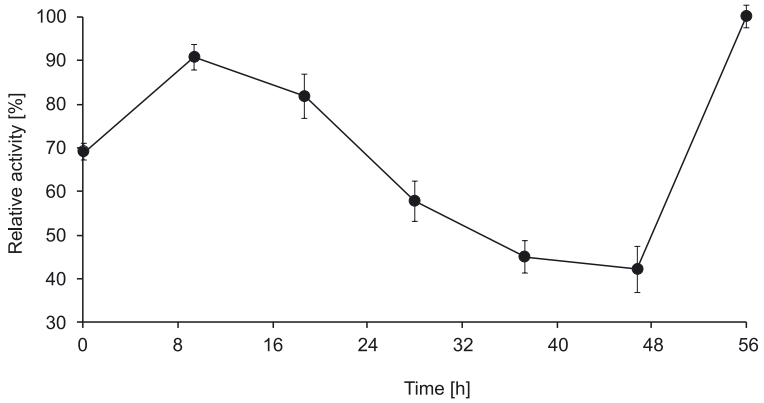

Fig. 6. Effect of starvation on collagenase activity of the midgut of *P. maculiventris*. The enzyme activity was restored after resuming the feeding of 40 h-starved bugs. Each value represents the mean of the triplicate measurements, and the vertical bars are the standard errors of the means

Starvation and collagenolytic activity

The effect of starvation on the midgut collagenase activity of *P. maculiventris* was shown in figure 6. The enzyme activity gradually decreased during starvation. There was up to a 50% reduction after a 40 h starvation, as compared to insects prior to the starvation treatment. The enzyme activity was restored after resuming the feeding of 40 h-starved bugs.

Discussion

The results showed maximum collagenolytic activity in the salivary gland and anterior midgut of *P. maculiventris*. The salivary glands' collagenase clearly is a major factor in the extra-oral digestion process and liquefaction of the internal host body's organs. Collagenase alongside serine proteases (Bell *et al*. 2005) are the main enzymes present in the salivary glands of the *P. maculiventris*. Optimal pH of total proteolytic activity of the salivary glands is associated with optimum pH of collagenase and serine proteases activity in the *P. maculiventris* (Bell *et al*. 2005) and other asopin species (Cohen 1995; Oliveira *et al*. 2006). Although the maximum collagenolytic activity in the midgut occurs at pH 8, the highest proteolytic activity in the different midgut parts corresponds to the activity of cysteine proteases. Bell *et al*. (2005) showed that maximum proteolytic activity of the midgut and salivary glands of *P. maculiventris* using the substrate azocasein, occurs at a pH of 6 and 8.5, respectively. They also emphasised that the major proteolytic activity in the midgut and salivary glands are associated with cysteine, protease and serine proteases, respectively. Bigham and Hosseininaveh's (2010) studies on proteases activity of the midgut of *Brachynema germari* (Pentatomidae) showed that hemoglobin is hydrolysed in a wide range of acidic and alkaline conditions (3–10) with an optimal pH of 5.

In the connective tissue, an extra cellular matrix is responsible for resistance against mechanical pressures (Alberts *et al*. 2007). In plant tissues a resistance to stretching

is provided by polysaccharides, especially pectin. In animal tissues, however, collagen fibers are responsible for stretch resistance. Collagen is the most abundant protein in animal bodies (Alberts *et al*. 2007), making up 25% of the total proteins in these organisms (Alberts *et al*. 2007). It is expected that carnivorous insects use enzymes as collagenase to exploit this rich source of protein. The enzyme has been studied in snakes, scorpions, and spiders, and it is known as an important component of venom (Hong and Chang 1985; Atkinson and Wright 1992).

Some studies on arthropods have indicated that collagenase is not sensitive to EDTA and so collagenase is to be considered a serine protease [e.g. the flesh flies, *Hypoderma lineatum* and *Lucilia cuprina* (Boulard and Garrone 1978; Bowles *et al*. 1988), the crab *Uca pugilator* (Eisen *et al*. 1973; Grant *et al*. 1983)]. Our results, however, revealed that the collagenase extracted from the salivary glands of *P. maculiventris* is sensitive to EDTA and so collagenase is a metalloproteinase. In line with our findings, Bell *et al*. (2005) showed that the salivary gland extract of *P. maculiventris* is affected by EDTA, implying that there is a presence of metalloproteinase in the salivary glands. A recent study on the closely related species *P. nigrispinus*, definitely revealed that collagenase is a metalloproteinase enzyme and it is one of the most important proteases in the salivary gland and midgut of the predatory bug (Fialho *et al*. 2012). High collagenase activity in the midgut of the predatory bugs may be explained by the fact that the enzyme contributes to the digestion of muscle fibers. Indeed, as a result of extra oral digestion (see the section Introduction), the damaged tissues, composed largely of small muscle fibers, enter the alimentary tract and are subsequently digested by high collagenase activity in the midgut (Fialho *et al*. 2012).

The results of time course experiments showed that collagenase activity is reduced while the predators went hungry. There is no such research on collagenase activity, though Bell *et al*. (2005) have shown that digestive protease activity in *P. maculiventris* is reduced by up to 90% after 96 h of starvation. A dramatic increase in collagenase activity in response to food ingestion after 40 h of starvation suggests collagenase secretion and production was strongly influenced by food signals. These signals can be free amino acids or other compounds generated during feeding (Barillas-Mury *et al*. 1995).

Host toxicity resulting from the saliva of predatory bugs can be due to the presence of digestive enzymes in the salivary glands (Babtist 1941). Following paralysis of the prey, host toxicity is enhanced by the destructive effects of digestive enzymes on the nervous system, muscle tissues and the host's internal organs (Babtist 1941). However, non-enzymatic toxic components in the saliva of some predatory bugs have been reported (Schmidt 1982; Corzo *et al*. 2001). It seems that different saliva components may have synergistic effects on each other. Among them, collagenase, by its effect on extra cellular matrix collagen fibers, plays an important role in the destruction of the host's internal tissues, and collagenase enhances the toxic effects.

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