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Biochemical Characterization of the Digestive Proteases in the Small Black and Yellow Wasp, *Allantus viennensis* Schr. (Hym.: Tenthredinidae)

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ABSTRACT

Knowledge on digestive proteases enzymes of insects needed for making plant expressing protease inhibitors to reach an alternative method to chemical control. In present study, biochemical properties of digestive proteases were determined in the alimentary canal of the small black and yellow wasp, *Allantus viennensis* Schr. (Hym.: Tenthredinidae) as important pest of Rose bushes. Larvae of *A. viennensis* were collected from rose plants in Rasht, Guilan province of Iran in summer (2016). Determining the proteolytic activity in gut of different larval instar of *A. viennensis* (2-5) showed that the enzyme activity increased with growing the larvae. The higher activity was found in the fifth instars larvae (7.46±0.06 $\mu mol^{-1}min^{-1}mg^{-1}$ protein). Also, comparison of proteolytic activities in different parts of digestive system of the fifth instars larvae showed that the enzyme activity in midgut was higher than that found in the foregut and hindgut. The optimal pH and temperature for enzyme activity in gut of fifth instars larvae were found at pH 10 and 30°C, respectively. Most inhibitory effect on the protease activity was obtained by PMSF as serine proteinases inhibitor (36.85%). The results of SDS-PAGE confirm the obtained data of inhibition assay. It showed that the serine proteinases are the major hydrolysing enzymes in the gut of larvae of *A. viennensis*.

Key words: Biochemical, inhibitor, protease, rose, small black and yellow wasp.

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INTRODUCTION

Small black and yellow wasp, *Allantus viennensis* Schr. (Hymenoptera: Tenthredinidae) is as important pest of rose plant in Guilan province (Iran). Initially, the young larvae feed on the parenchyma of the youngest leaves and as larvae grow, they eventually eat the entire leaf except main rib. Rose flower petals, shoots and stem can also damage by larvae of the pest (Hosseini & Sahragard, 2003). Chemical control on the pest is not advisable due to planting roses in urban areas, so development of alternative methods to chemical control is necessary to decrease the harmful effects. Proteases are very important enzymes in insects that hydrolyze the peptide bonds in dietary proteins to liberate the amino acids needed for growth and development, and inactivate protein toxins ingested as a consequence of feeding (Terra, Ferreira, Jordao, & Dillon, 1996). Serine, cysteine (thiol), aspartic (carboxyl), and metalloproteases are classes of proteases (Barrett, 1986). Protease inhibitors are proteins or polypeptides which bind with proteolytic enzymes may interfere with insect's normal digestive physiology disrupting digestion and reducing growth and survival (Gatehouse, Gatehouse, & Brown, 2000). These inhibitors present in plants and provide natural defense against herbivorous insects can use for producing transgenic plants resistant to pests. To reach this goal, at first it is necessary to characterize the digestive protease enzymes present in an insect. So far, biochemical properties of proteases were studied from the digestive system of many insect orders (Sharifi, Ghadamyari, Gholamzadeh-Chitgar, & Ajamhassani, 2012a; Gholamzadeh-Chitgar, Ghadamyari, & Sharifi, 2013) but there is a little information on Hymenoptera (Jany, Haung, & Ishay, 1978; Sharifi, Gholamzadeh-Chitgar, Ghadamyari, Sajedi, & Amini, 2012b). In this research we study the biochemical properties of digestive proteases of *A. viennensis* and the effects of various inhibitors on enzyme activities to find a new method for control of the pest.

MATERIAL AND METHODS

Insects and gut enzyme preparation

Larvae of *A. viennensis* were collected from rose plants in Rasht, Guilan province of Iran in summer (2016). The population maintained on rose leaves in optimum rearing conditions of 25 ± 2°C, 60% ± 10 RH with a photoperiod of 16 h light and 8 h dark. For enzyme preparation, larvae were anaesthetized on ice and alimentary canal of different larval instars (2^{nd} to 5^{th}) and also three parts of gut: foregut, midgut and hindgut in 5^{th} larval instars were removed. The samples were homogenized in a known volume of distilled water. The crude gut homogenate was centrifuged at 13,000 rpm for 10 min at 4°C (Sharifi et al, 2012b). The supernatant was used as an enzyme source.

Protease activity measuring

Protease assay was carried out as described by Sharifi et al (2012b) with some modifications. Using azocasein 2.5% as substrate the total protease activity was determined. 10 µl enzyme was added to 48 µl universal buffer (50 mM sodium acetate-phosphate-glycine) with the desired pH (pH=10). After 5 min 18 µl substrate

was added. The reaction mixture was incubated at 35°C for 60 min. Proteolysis was stopped by addition of 50 µl of 30% trichloroacetic acid (TCA). After cooling at 4°C for 30 min, samples were centrifuged at 13000 rpm for 10 min. Then an equal volume of 1 N NaOH was added to the supernatant and the absorbance was recorded at 450 nm (microplate reader, Awareness Technology Inc., Stat Fax® 3200).

Tryptic and chymotryptic activity

Tryptic activity was assayed using 1 mM BApNA (N-benzoyl-L-arg-p-nitroanilide) as substrate. 10 µl enzyme, 85 µl of 25 mM acetate-phosphate-glycine buffer (pH=10) and 5 µl substrate was used. The absorbance was read at 405 nm continuously monitoring the change in absorbance p-nitroaniline release for 10 min at 25°C with a microplate reader (Gholamzadeh-Chitgar et al, 2013).

Chymotryptic activity measured using 1 mM BTEE (benzoyl-L-tyrosine ethyl ester) as substrate according to Hummel (1959). The substrate dissolved in 50 % methanol (v/v), and in 0.08 M Tris-HCl (pH 7.8) containing 0.1 M $CaCl_2$ at room temperature. The increase in absorbance at 256 nm due to the hydrolysis of the substrate was recorded by monitoring the absorption at the wave length.

Effect of pH and temperature on enzyme activity

The optimum pH for general protease activity (azocasein as substrate) and specific proteolytic activity (BApNA as substrate) was determined using sodium acetate-phosphate-glycine buffer ranging from pH 3 to 12. The temperature range from 20 to 70°C was used to find optimal temperature for general proteolytic activity. Enzyme activity was measured by the standard assay method mentioned above (Sharifi et al, 2012b).

Effects of inhibitors on protease activity

PMSF (phenyl methane sulfonyl floride, 5mM); TLCK (N-p-tosyl-L-lys chloromethyl ketone, 1mM); TPCK (N-tosyl-L-phe chloromethyl ketone ,1mM); EDTA (ethylene diamine tetraacetic acid, 2mM), Iodoacetate and Iodoacetic acid (5 mM) used for determining the effect of inhibitors on proteolytic activities. 10 µl of different inhibitors and 15 µl of enzyme were incubated at 35°C for 10 min. Then 33 µl of sodium acetate-phosphate-glycine buffer with the desired pH was added. Then protease activity was measured as aforementioned in the section of protease assays (Sharifi et al, 2012b).

Determination of protein concentration

Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Zymogram analysis

Electrophoresis of proteolytic enzyme was performed according to Laemmli (1970). A total of 24 µl of the enzyme extract was mixed with 10 µl of inhibitor solution. After incubation for 30 min in room temperature, 10 µl of sample buffer was added. Then the samples were loaded into the wells of each polyacrylamide substrate gel and

electrophoresis was carried out at 4°C in a constant voltage of 100 V. After the run, the gel was removed and placed in phosphate buffer containing 2.5% Triton X-100 for 20 min. After this step, the gel was immersed in 0.5-1% casein and shacked for 3 h. Then, the gel was washed in distilled water and stained with 0.1% Coomassie brilliant blue R-250 in methanol-acetic acid-water (50:10:40). After 2 h, the gel was washed in water and destaining was done in methanol-acetic acid-water (50:10:40) for 1-2 h until clear bands could be visualized against a dark blue background.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) (SAS Institute Inc., 2002). Differences between sample (n= 3) means were evaluated using Tukey's test ($p \leq 0.05$).

RESULTS

The results clearly revealed presence of proteases in digestive system of larvae of *A. viennensis*. Determining the proteolytic activity in gut of different larval instars of *A. viennensis* showed that the enzyme activity increased with growing the larvae (Fig. 1A). The higher activity was found in the fifth instars larvae (7.46±0.06 $\mu mol^{-1}min^{-1}mg^{-1}$ protein). By comparison of proteolytic activities in different parts of digestive system of the fifth instars larvae, the enzyme activity in midgut was higher than that found in the foregut and hindgut (Fig. 1B).

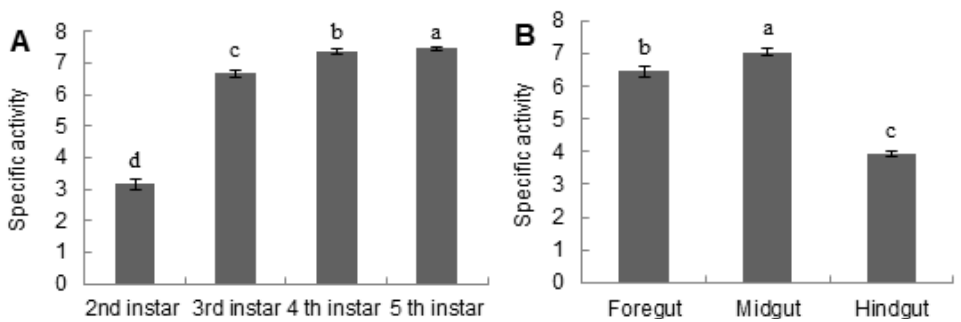

Fig. 1. Total proteolytic specific activity (μmol/min/mg protein) in gut of different larval instars (A) and three parts of digestive system of the fifth instars larvae (B) of *Allantus viennensis.* Means followed by the different letters are significantly different by Tukey's test ($p < 0.05$).

The presence of trypsin- and chymotrypsin-like proteases have been shown in larval digestive extracts by using BAPNA and BTEE as specific substrates. The trypsin and chymotrypsin activity were 0.394±0.16 and 1.70 ± 0.03 $\mu mol^{-1}min^{-1}mg^{-1}$ protein, respectively.

The optimal pH for enzyme activity in gut of fifth instars larvae was found at pH 10 (Fig. 2A). Protease activity increased gradually from pH 3 to 10 and reached to a maximum at pH 10 then fell. Trypsin showed higher activity in alkaline pH and optimal pH in the gut of larvae of *A. viennensis* was 11 (Fig. 2B).

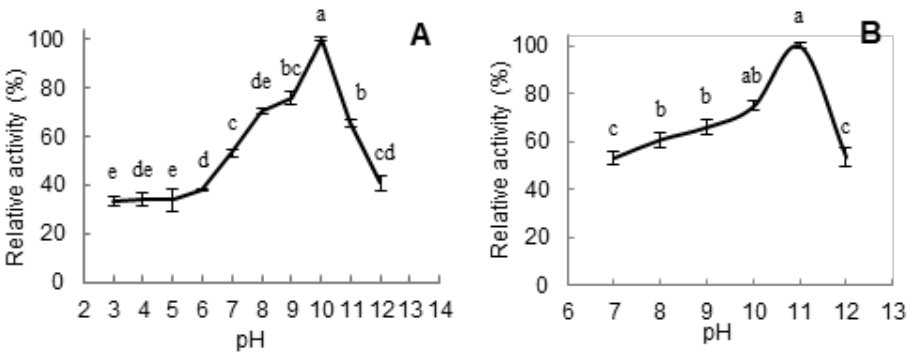

Fig. 2. Effect of pH on the proteolytic (A) and trypsin (B) activities of gut extract from 5th larval instars of *Allantus viennensis*. Means followed by the different letters are significantly different by Tukey's test ($p < 0.05$).

The optimal temperature for proteolytic activity in the gut of *A. viennensis* was 30°C. Enzyme activity increased by increasing temperatures to reach maximal activity at 30°C and then fall to 21% at 70°C (Fig. 3).

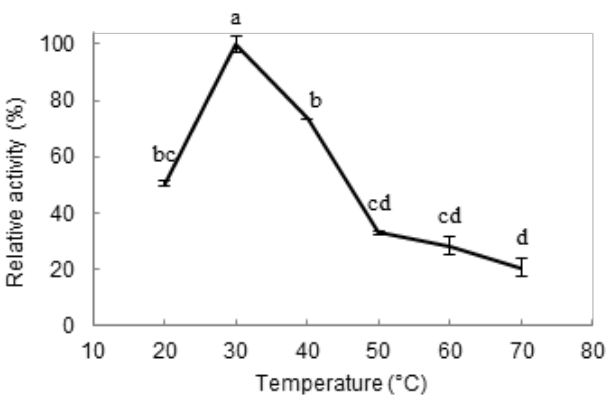

Fig. 3. Effect of temperature on the proteolytic activity of gut extract from 5th larval instars of *Allantus viennensis*. Means followed by the different letters are significantly different by Tukey's test ($p < 0.05$).

Various proteinase inhibitors showed significant differences on the enzyme activity compared with the control (Fig. 4). Most inhibitory effect on the protease activity was obtained by PMSF (36.85%). Also, TLCK (Trypsin-like serine proteases inhibitor), TPCK (Chymotrypsin-like serine proteases inhibitor), Iodoacetate, Iodoacetic acid (Cysteine proteases inhibitors) and EDTA (Metalloproteases inhibitor) were decreased the enzyme activity 20.89, 18.57, 17.91, 17.41 and 16.79% respectively.

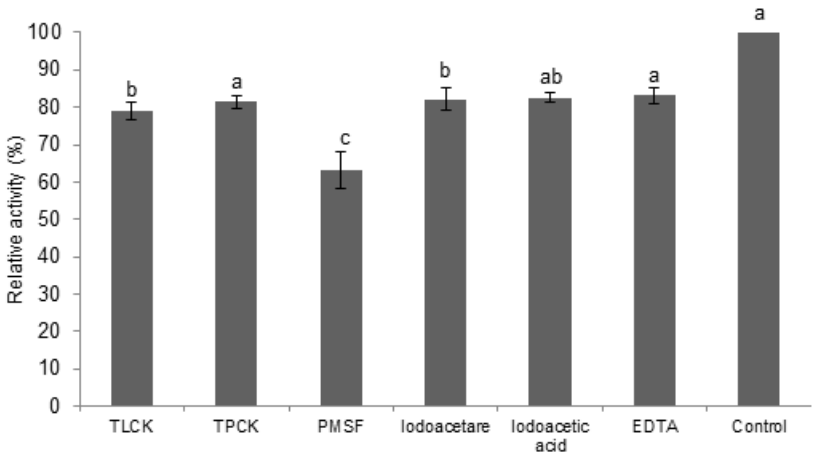

Fig. 4. Effect of some proteinase inhibitors on the proteolytic activity of gut extract from 5th larval instars of *Allantus viennensis.* Means followed by the different letters are significantly different by Tukey's test ($p < 0.05$).

As shown in the figure 5 at least four protease bands, namely P1, P2, P3 and P4 for control were detected by Electrophoresis. The results of SDS-PAGE confirm the obtained data of inhibition assay. According to the results PMSF reduced intensity of the bands compared to the control in the gel electrophoresis zymogram.

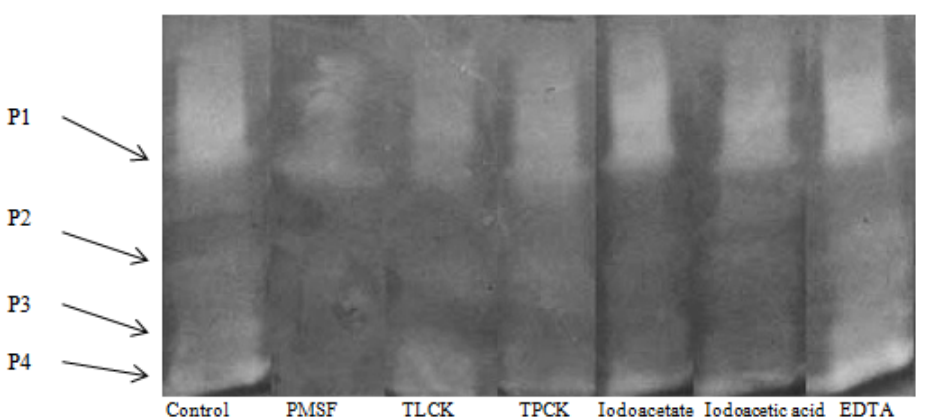

Fig. 5. Effect of some proteinase inhibitors on the proteolytic activity of gut extract from 5th larval instars of *Allantus viennensis*.

DISCUSSION

In the current study the digestive protease enzyme of *A. viennensis* was characterized for the first time. According to the obtained results the protease enzymes

are presented in the gut of larvae. The most enzyme activity was found in the fifth instars larvae. It is reported that there is a relation between food absorption and the enzyme activity. The more enzyme activity can be occurred with increasing the food absorption (Christopher & Mathavan, 1985). In *A. viennensis*, the enzyme had the highest activity in the midgut than the foregut and hindgut. The midgut is the principal source of digestive enzymes and also one of the main sites for the absorption of digested material (Vazquez, Smith, Martnez-Gallardo, Blanco-Labra, 1999). A similar result was observed in *A. viennensis* when the most α-amylase and α- β galactosidases activities were obtained in 5th larval instar and in midgut (Jahanjou, Gholamzadeh-Chitgar, Ghadamyari, & Hosseini, 2018). Same conclusion was reported by Sharifi et al (2012b) in the rose sawfly, *Arge rosae* L. (Hymenoptera: Argidae). According to the trypsin and chymotrypsin activities results, the values are lower than that reported for *A. rosae* that show the low activities of them in gut of *A. viennensis* (Sharifi et al, 2012b). However, the presence of trypsin-like and chymotrypsin-like enzymes demonstrates an insect's ability to access structural or other insoluble proteins (Cohen, 2000).

Protease activity in the gut of *A. viennensis* was active more than 70% at pH 8-10. It shows the enzyme had maximum activity in alkaline conditions. The pH of gut contents is a major factor that affects digestive enzymes (Terra & Ferreira, 1994). In alkaline environment serine proteases such as trypsin, chymotrypsin and elastase are most active (Christeller, Liang, Markwick, & Burgess, 1992). In our study, according to the inhibition assay and zymogram analysis results, the type of protease in the gut of *A. viennensis* was detected as serine proteases. This finding is consistent with those reported for serine proteases that they are generally active at neutral and alkaline pH, with an optimum pH between 7-11 (Ellaiah, Srinivasulu, & Adinarayana, 2002). The high pH of the gut attributed to an adaptation of herbivorous larvae for releasing hemicellulose from plant cell walls. Alkaline proteases are a physiologically important group of enzymes and play a specific catalytic role in the hydrolysis of proteins (Ellaiah et al, 2002). Surveys show that midgut pH is a species-specific trait and is generally conserved within major insect orders as well (Berenbaum, 1980; Keating, Schultz, & Yendol, 1990). The high optimal pH of the proteolytic activities in the gut of *A. viennensis* is in agreement with those reported for other hymenopteran serine proteases (Wolfson & Murdock, 1990; Sharifi et al, 2012b).

Protease activity in the gut of *A. viennensis* increased from temperature 20°C to optimal value (30°C) then decreased. Biological reactions occur faster by increasing temperature up to the point of enzyme denaturation, above which temperature, enzyme activity and the rate of the reaction decreases sharply (Zibaee & Fazeli-Dinan, 2012). In case of temperature, obtained value is similar to finding on gut extracts of *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) (Al Jabr and Abo-El-Saad, 2008) and *Achaea janata* L. (Lepidoptera: Erebidae) (Budatha, Meur, & Datta-Gupta, 2008).

In this study, PMSF as the serine-protease inhibitor caused significant decrease than other inhibitors on proteolytic activity in the gut of *A. viennensis*. This result showed that the serine proteinases are the major hydrolysing enzymes in the gut of the pest. Similar results in the case of Hymenoptera order was reported by Down et

al (1999) on ectoparasitoid *Eulophus pennicornis* Ness and Burgess & Gatehouse (1997) in gut extract of the honeybee, *Apis mellifera* L.. Also, Sharifi et al (2012b) found that PMSF had the greatest inhibition effect on proteolytic activity in *A. rosae* demonstrating the serine proteinases as dominant enzymes in the gut. Slight inhibition of protease activity occurred by EDTA suggesting that Metalloproteases were slightly responsible for protein digestion in the gut of *A. viennensis*.

In the gel electrophoresis zymogram, PMSF reduced intensity of the bands compared to the other inhibitors. The proteinase inhibitor revealed strong inhibition of P2, P3 and P4 in the gel electrophoresis zymogram. The data resulting from inhibition assay by PMSF strongly confirmed this finding. It revealed the presence of serine proteases as the major proteases in the gut of *A. viennensis*. Because, proteases have a reactive serine residue in the active site and are generally inhibited by PMSF (Ellaiah et al, 2002). Similarly, Hegedus et al (2003) and George, Ferry, Beak, & Gatehouse (2008) found that PMSF reduced proteolytic activity in the gut of lepidopterus pests: *Mamestra configurata* Walker and *Busseola fusca* Fuller, respectively. In the gut of *Osphranteria coerulescens* Red. (Coleoptera: Cerambycidae) similar result was reported by Sharifi et al (2012a).

CONCLUSION

The results of the present study revealed that the protease enzyme present in gut of *A. viennensis* larvae. The maximum enzyme activity was obtained at pH 10 and 30°C. Also, serine proteinases were dominant protease in the gut of this pest. The results of this study provide knowledge needed for making plant expressing protease inhibitors to the control of *A. viennensis*.

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