IDENTIFICATION AND CHARACTERIZATION OF MIDGUT DIGESTIVE PROTEASES FROM THE ROSACEOUS BRANCH BORER, *OSPHRANTERIA COERULESCENS* REDTENBACHER (COLEOPTERA: CERAMBYCIDAE)

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Larval midgut extracts from rosaceous branch borer, *Osphranteria coerulescens,* were assayed for protease activity in the presence of protease inhibitors in order to determine the dominant protease types. The maximum total proteolytic activity in the midgut extract using azocasein as substrate was observed at pH 8, suggesting the presence of serine proteases. Also, the highest activity using azocasein as substrate was revealed at temperature 55°C. The presence of serine proteases was confirmed by their noticeable inhibition due to specific inhibitor, PMSF. The significant inhibition of tryptic activity by the inhibitors compared to chymotrypsin showed a considerable presence of trypsin in the midgut. Also, inhibition assays and zymogram analysis pointed to the presence of metalloprotease in the digestive system of *O. coerulescens*. The kinetic parameters of trypsin-like proteases, using BAPNA as substrate at pH 8 and temperature 25°C, indicated that the K_m and V_{max} values of trypsin in the gut of last instar larvae were 0.69±0.01 mM and 560±2 nmol.min^{-1}mg^{-1}protein, respectively. Inhibition assays and zymogram analysis showed that only small amounts of chymotrypsin and cysteine proteases were present in the digestive system of *O. coerulescens*, with metalloproteases and trypsin being dominant.

Keywords: *Osphranteria coerulescens*, chymotrypsin, trypsin, protease inhibitor.

INTRODUCTION

Insects generally use different types of digestive enzymes secreted by midgut's epithelial cells (1, 2, 3). These enzymes allow them to digest a wide range of food diets (4) including polymeric molecules. Some carbohydrases and proteases can break down the molecules into absorbable elements in midgut (5).

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Proteases are hydrolytic enzymes present in digestive system and hemolymph of insects that plays important roles as they which are involved in food digestion, polyphenoloxidase activation, liberation of amino acid needed for growth, toxin activation/detoxification, and inflammation processes (6). The insect proteinases are classified based on their catalysis mechanisms as serine proteinases, cysteine proteinases, aspartic proteinases, and metalloproteinases (7). However, the expression level of each different type of proteases in insect guts depends on ingested protein content (8). Inhibition of these proteases may lead to an increase in plant defenses against insects and pathogens. Therefore, proteinase inhibitors have been considered as a safe alternative method against herbivorous pests, because they cause interruption in proteolytic activity and retard in the larval growth and development of some insect species including Coleoptera (9).

The rosaceous branch borer, *Osphranteria coerulescens* Redtenbacher (Coleoptera: Cerambycidae) is a key pest on rosaceous plants causing significant damage to living trees in Iran. Larvae of the insect feed on living twigs and branches of various Rosaceae and in high population density can cause heavy damage to fruit trees. Currently, control of the pest in Iran relies exclusively on the use of systemic and semi-systemic pesticides. Control of the rosaceous branch borer is notoriously difficult because the pest larvae live inside the twigs and branches during their entire larval and pupae life cycles. Therefore, it makes the control difficult using chemical pesticides. However, the regular and repeated use of one particular chemical pesticide results not only to pesticide resistance but also impairs the balance between pests populations and those of their natural enemies (10) and overall to low environmental quality. Therefore, an integrated pest management (IPM) program, including application of selective pesticides, pheromone, some protease inhibitors beside as well as the use of crop rotation and field sanitation would provide the best management option for control of this pest (11).

Protease inhibitors, as natural products in some plant such as legumes, are potential controlling agents against herbivorous pests (12). Different types of protease inhibitors inhibit the proteolytic activity in the alimentary canal of insect, and cause to retard insect growth and development (12). Therefore, the first step in the production of transgenic plants containing specific protease inhibitor is the biochemical study of these enzymes. Also, identification protease types and their characterization in insect digestive system are necessary for identification of natural proteinous inhibitors which can be used to control populations of herbivorous pests (13). For an efficient control of *O. coerulescens* through this strategy, it is necessary to identify the type of protease present in the gut of the pest.

Our information on the midgut proteases of *O. coerulescens* is insufficient and the current study presents biochemical properties of digestive proteases of the

pest and the effects of various enzyme inhibitors on enzyme activities with the aim of identification and application of new pest management technologies.

MATERIALS AND METHODS

CHEMICALS

Azocasein, BApNA (N-benzoyl-L-arg-p-nitroanilide), BTEE (benzoyl-L-tyrosine ethyl ester), TLCK (N-p-tosyl-L-lys chloromethyl ketone), TPCK (N-tosyl-L-phe chloromethyl ketone), PMSF (phenyl methane sulfonyl floride) and Iodoacetate were purchased from Sigma (USA). Trichloroacetic acid and EDTA (ethylene diamine tetraacetic acid) were obtained from Merck Company (Germany).

INSECTS

O. coerulescens larvae were collected from *Rosa* sp. in the national botanic park, Research Institute of Forests and Rangelands, Karaj- Iran. The last larval instar was selected for measuring enzyme activity.

PREPARATION OF ENZYME EXTRACT

The last larval instar of *O. coerulescens* was randomly selected for measuring enzyme activity. Larvae were anaesthetized on ice, their whole guts were removed (Fig. 1) and stored at -20 °C until further use. The sample was homogenized in a known volume of distilled water for measuring the optimum pH. The sample for measuring specific activity and temperature effect on enzyme activity was prepared in buffer (optimum pH for each enzyme). The crude gut homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was used as enzyme solution.

PROTEASE ASSAYS

Protease activity was spectrophotometrically assayed according to Garcı´a-Carren *et al*. (14) with some modifications. The total protease activity was determined using azocasein 2.5% as substrate. For azocaseinolytic activity, the reaction mixture consisted of 15 µl enzyme, 43 µl universal buffer, 50 mM sodium acetate-phosphate-glycine, with the desired pH (pH=8). After 5 minutes, 17 µl substrate were added. The reaction mixture was incubated at 35 °C for 90 minutes. Proteolysis was stopped by addition of 50 µl of 30% trichloroacetic acid (TCA). After cooling at 4 °C for 30 minutes, samples were centrifuged at 13000 rpm for

10 minutes. Then, an equal volume of 1 N NaOH was added to the supernatant and the absorbance was recorded at 440 nm (with a microplate reader, Awareness Technology Inc., Stat Fax® 3200). Absorbance was then converted to units of protease activity by the following equation: (absorbance/extinction coefficient) × 103= micromoles of dye. Protease activity was expressed as µmol dye/min/mg protein.

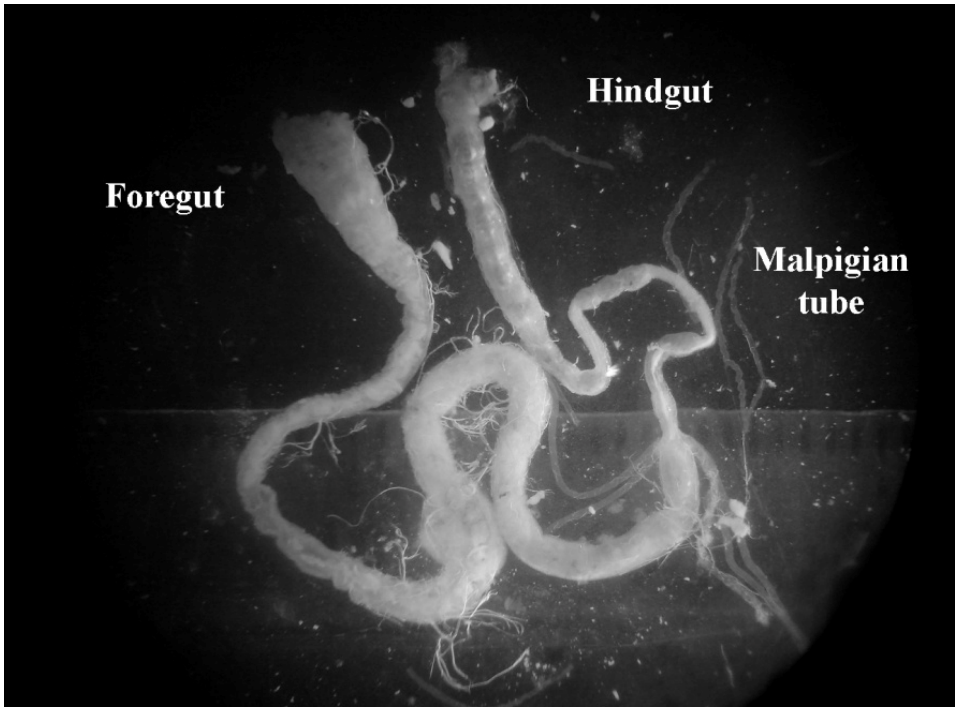

Fig. 1 – Digestive system of *O. coerulescens*.
*Larvae were dissected in cold saline buffer under a light microscope and photographed.

Tryptic activity was assayed using 1 mM BApNA as substrate. 10 µl enzyme, 85 µl of 25 mM acetate-phosphate-glycine buffer (pH=8) and 5 µl substrate were used. Absorbance was read at 405 nm, continuously monitoring the change in absorbance of p-nitroaniline release for 10 min at 25 °C with a microplate reader.

Chymotryptic activity was measured using 1 mM BTEE as substrate, according to Hummel (15). The substrate dissolved in 50% methanol (v/v) and in 0.08 M Tris-HCl (pH 7.8) containing 0.1 M CaCl2 at room temperature (25 °C). The increase in absorbance at 256 nm due to the hydrolysis of the substrate was recorded by monitoring the absorption at the wavelength. Assays aforementioned were carried out in triplicate, and for all of them, appropriate blanks were run.

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 pH 12. A temperature range from 15 °C to 75 °C was used to find the optimal value for general proteolytic activity in the gut of *O. coerulescens*. Assays were carried out as previously described in protease assays section.

EFFECTS OF INHIBITORS ON PROTEASE ACTIVITY

Proteolytic activities in gut extracts of *O. coerulescens* were assayed in the presence of specific protease inhibitors, including PMSF (5 mM); TLCK (1 mM); TPCK (1 mM); EDTA (2 mM), Iodoacetate (5 mM). 10 µl of different inhibitors and 15 µl of enzyme were incubated at 35 °C for 15 minutes. Then, 33 µl sodium acetate-phosphate-glycine buffer with the desired pH were added. The rest of the steps were similar to those aforementioned in the of protease assays section. All inhibition assays were carried out in three replicates.

DETERMINATION OF PROTEIN CONCENTRATION

Protein concentration was estimated by the method of Bradford (16) using bovine serum albumin (BSA) as the standard.

TRYPSIN K_m And V_{max}

The Michaelis–Menten constant (K_m) and the maximal reaction velocities (V_{max}) of trypsin were investigated at different concentrations of BApNA over the range 0.0156-1 mM (final concentration), in 20 mM Tris-HCl. The experiment was performed at pH 8 and 25 °C. K_m and V_{max} were estimated from the Lineweaver–Burk plots.

ELECTROPHORESIS ZYMOGRAM

Protease activity in the electrophoretic gel (17) was determined using resolving and stacking polyacrylamide gels of 12.5% (w/v) and 5% (w/v), respectively. A total of 10 µl of the enzyme extract was mixed with 10 µl of inhibitor stock solution. After incubation for 20 minutes at room temperature, 8 µl of sample buffer without mercaptoethanol were added, and the samples were loaded into the wells of each polyacrylamide substrate gel. Non-denaturing polyacrylamide gel was carried out at 4 °C in a constant 100 V voltage. After the run, the gel was removed and placed in phosphate buffer pH 8 containing 2.5%

Triton X-100 for 20 minutes. Afterwards, the gel was immersed in 0.5-1% casein and shaken for 3 hours, then it 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 hours, the gel was washed in water and destaining was done in methanol–acetic acid–water (50:10:40) for one to two hours, until clear bands could be visualized against a dark blue background. Any proteolytic activity was evident as cleared bands within the gel.

STATISTICAL ANALYSIS

Data were subjected to analysis of variance (ANOVA), and the means were compared by Tukey's test. Statistical analyses were performed at P = 0.05 using the software SAS (18).

RESULTS

PROTEASE ACTIVITY

The proteolytic activity found in the gut of *O. coerulescens* when using azocasein as a protein substrate was 9.12±0.03 μmol $min^{-1}mg^{-1}$ protein. Trypsin activity (in acetate-phosphate-glycine buffer, pH 8 and 25 °C) and chymotrypsin activity (in tris- HCl buffer, pH 7.8 and 25 °C) were 1.58±0.16 and 0.612±0.01 μmol $min^{-1}mg^{-1}$ protein, respectively.

EFFECT OF pH AND TEMPERATURE ON ENZYME ACTIVITY

The pH dependence of proteolytic activity with azocasein as a protein substrate in the gut extract from *O. coerulescens* larvae is illustrated in Fig. 2. Proteolytic activity showed that the substrate was hydrolyzed over a broad range of pH values, with a peak of maximum activity at pH 8. Proteases from the gut of this insect retained more than 70% of activity at pH 7-9. Trypsin was more active at alkaline pH, its optimal pH in the gut of *O. coerulescens* larvae being 8 (Fig. 3). As seen in Fig. 4, the optimal temperature for proteolytic activity in the gut of *O. coerulescens* using azocasein was 55 °C.

KINETIC PARAMETERS

Kinetic analysis of trypsin-like activity at pH 8 gave linear reciprocal Michaelis–Menten (Lineweaver–Burk) plots, enabling estimation of values for K_m and V_{max}. Kinetic parameters of trypsin in the gut of larvae were measured by BAPNA as substrate. The K_m and V_{max} values of trypsin in the gut were 0.69±0.01 mM and 560±2 $nmol.min^{-1}mg^{-1}$protein, respectively (Fig. 5).

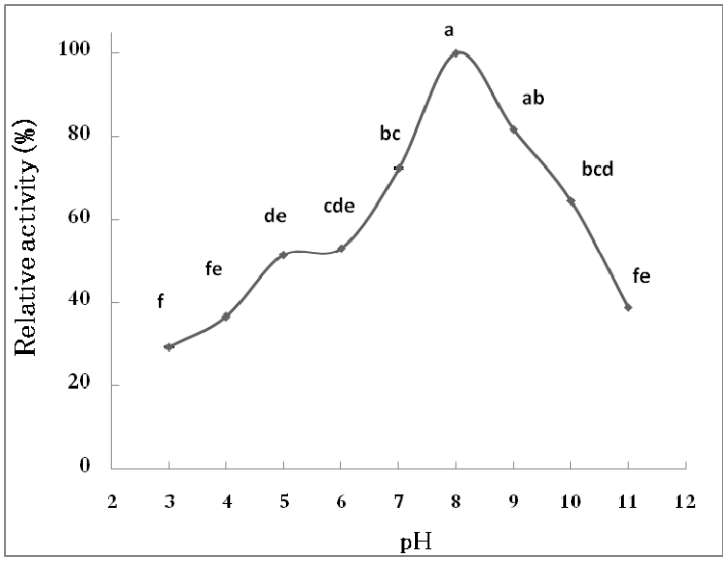

Fig. 2 – Effect of pH on the total proteolytic activity of larval gut extract from *O. coerulescens* using azocasein as substrate.
*Different letters indicate that the activity of enzyme in different pH are significantly different from each other by Tukey's test (p <0.05).

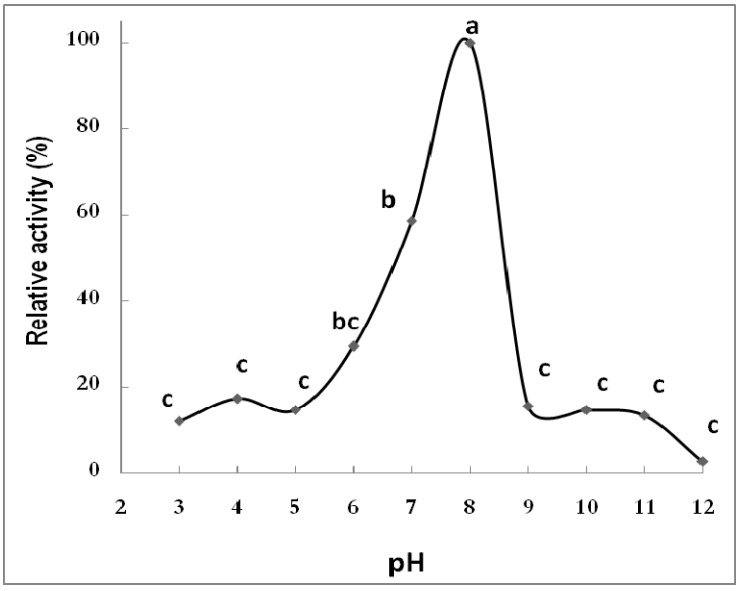

Fig. 3 – Effect of pH on trypsin relative activity (%) extracted from gut of *O. coerulescens* larvae using BApNA as substrate.
*Different letters indicate that the activity of enzyme in different pH are significantly different from each other by Tukey's test (p <0.05).

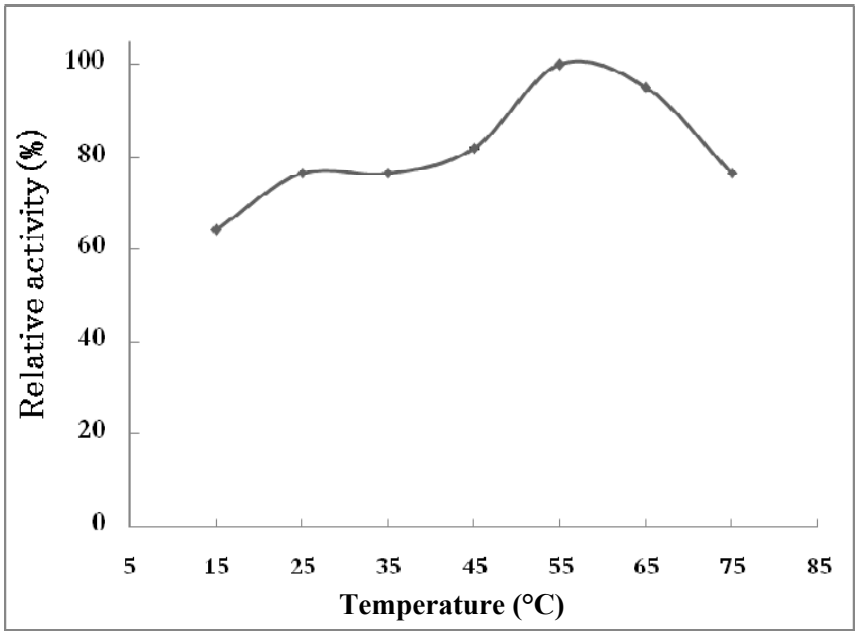

Fig. 4 – Effect of temperature on the activity of gut total proteases in *O. coerulescens* using azocasein as substrate.

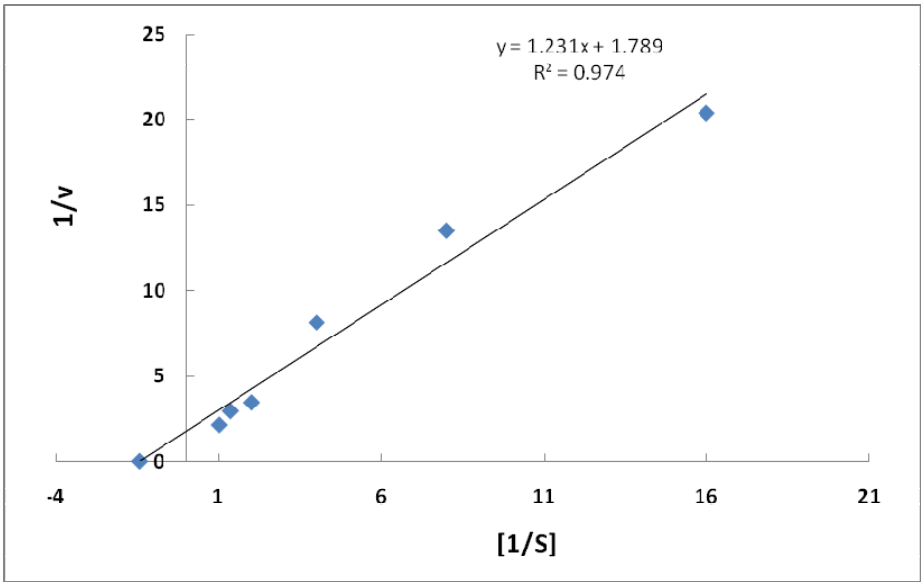

Fig. 5 – Double reciprocal plot showing the kinetic parameters of trypsin from the gut of *O. coerulescens* larvae using BApNA as substrate.

ENZYME INHIBITION ASSAYS

The effect of various inhibitors on protease activity is shown in Table 1. Results showed that PMSF (5 mM) inhibited 80.06% of proteolytic activity in the gut of *O. coerulescens*. Comparatively to other inhibitors, PMSF (serine protease inhibitor) caused considerable inhibition of proteolytic activity, suggesting that serine proteases are the major proteolytic enzymes of *O. coerulescens* gut. Also, EDTA (metalloprotease inhibitor) caused a significant inhibitory effect (58.9%) on hydrolyzing azocasein, suggesting that metalloproteases, moreover serine proteases, play the most important roles in protein digestion in the gut of *O. coerulescens*.

Table 1

Effect of some proteinase inhibitors on the gut protease activity in *O. coerulescens* larvae

Inhibitor	**% Inhibition (Mean ±SE)**	**Target proteases**	**Final concentration**
PMSF	80.06±0.2	Serine proteases	5 mM
TLCK	67.9±0.6	Trypsin-like serine proteases	1 mM
EDTA	58.9±0.2	Metalloproteases	2 mM
TPCK	32.09±0.18	Chymotrypsin-like serine proteases	1 mM
Iodoacetate	23.7±0.8	Cysteine proteases	5 mM
Control	0	-	-

ZYMOGRAM ANALYSIS

The effect of proteinase inhibitors on proteolytic activity in gel is shown in Fig. 6. Crude *O. coerulescens* extracts were analyzed by SDS-PAGE. After protease activity staining, at least 5 major bands were detected based on their electrophoretic mobility. The results of SDS-PAGE confirm the effects of inhibitors in their absence and presence, respectively, in the inhibition assay. However, as seen in Fig. 6, PMSF caused complete disappearance of bands p1, p2, p3, and a reduced intensity of p4 band compared to the control. According to this result, the zymogram (made using casein as substrate) clearly showed that trypsin was the main protease in the gut of *O. coerulescens*.

DISCUSSION

Midgut is the most important source of digestive enzymes in *Coleoptera* as well as one of the main sites for the absorption of digested material (19). We found that gut extracts of *O. coerulescens* larvae had an optimum azocaseinolytic proteinaceous activity at pH 8. Proteases from this insect gut are active at a broad pH range. The enzyme retained about 30-50% of activity in acidic conditions (pH 3-6)

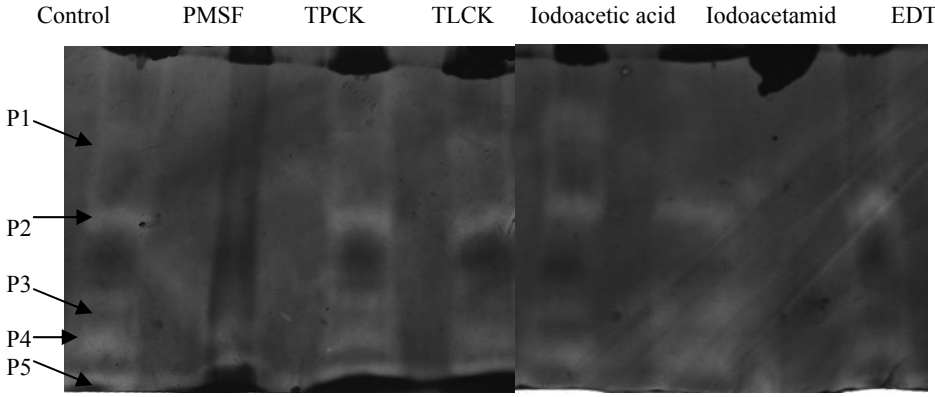

Fig. 6. – Effect of some proteinase inhibitors on azocasein hydrolytic activity of digestive system extract in 12.5% non-reducing SDS-PAGE. 1: Iodoacetic acid (10 mM), 2: EDTA (4 mM), 3: PMSF (10 mM), 4: TLCK (2 mM), 5: TPCK (2 mM), 6: Control.
*Each alimentary canal homogenized in 10 µl tris-HCl buffer (pH 8.0) and 10 µl of the enzyme extract was mixed with 10 µl of inhibitor stock solution.

and more than 70% at pH 7-9, with a peak of maximum activity at pH 8. Alkaline pH values for activity are due to intrinsic alkaline pH of insect digestive system; similar data have been previously reported for some coleopteran insects (20, 21). In this study, the high optimal pH of proteolytic activities from *O. coerulescens* gut is in agreement with those reported for grass grub larvae, *Costelytra zealandica* (*Coleoptera: Scarabaeidae*), the physiological pH of the midgut being 9-10 (20). Also, Biggs and McGregor (22) found that gut extracts of *C. zealandica* larvae had an optimum azocaseinolytic proteinaceous activity at alkaline pH. The optimal pH of the total proteolytic activity in the posterior midgut of *Tenebrio molitor* (*Coleoptera: Tenebrionidae*) larvae was found to be 9.0 (23). Maximum levels of proteinase activity in the larval midguts of a common poplar tree borer, *Anoplophora glabripennis Motsch* (*Coleoptera: Cerambycidae*), were reported at pH 10-11.5 (24). The highest protease activity was found in *Trogoderma granarium* (*Coleoptera: Dermestidae*) (25) and *Lasioderma serricorne* (*Coleoptera: Anobiidae*) (26) at pH 8-11 and 8.5-9.0, respectively. Other studies reported an optimum proteolytic activity at acidic pH in the gut of some coleopteran insects. Such discrepancies between midgut pH values may be related to the different feeding habits. For example, some members of the *Dermestidae* family, such as *Attagenus megatoma* (*Brahm*) were shown to have an alkaline pH in their midguts (27), while *Dermestes maculatus* (*Coleoptera: Dermestidae*) larvae had slightly acidic pH in their midgut (28), which may be due to the fact that dermestids feed on a wide variety of materials of both animal and plant origin. Wieman and Nielsen (29) found that the pH range for optimum activity in the gut of *Acanthoscelides obtecus* (*Coleoptera: Tenebrionidae*) was 4.5-7.0. Optimal casein hydrolysis at pH 6.2, by gut extracts from *Cynaeus angustus* (*Coleoptera:*

Tenebrionidae), was reported (30). Similar results were obtained by Vazquez *et al*. in their research on *Prostephanus truncatus* (*Coleoptera: Bostrichidae*) (19).

Digestive system extracts of *O. coerulescens* have azocaseinolytic activity within a broad range of temperatures (15-75°C), with an optimum at 55°C. Young *et al*. (31) found that gut extracts of dung beetles, *Catharsius molossus* (*Coleoptera: Scarabaeidae*), have an optimum azocaseinolytic proteinaceous activity at 40°C. A higher enzyme activity in gut extracts of *Rhynchophorus ferrugineus* (*Coleoptera: Curculionidae*) was observed by increasing temperatures to reach maximal activity at 30°C (32). Trypsin showed higher activity at alkaline pH, its optimal pH in the gut of *O. coerulescens* larvae being 8 (Fig. 3). Hosseininaveh *et al*. (25) showed that the substrate BApNA was hydrolyzed by *T. granarium* trypsin at alkaline pH, with maximum activity at pH 9-10 and almost no activity at acidic pHs. According to Ortego *et al*. (33), maximal hydrolysis of BApNA by trypsin in the digestive system of *Aubeonymus mariaefranciscae* (*Coleoptera: Curculionidae*) occurred at pH 11.5.

Trypsin (BApNA as substrate) and chymotrypsin (BTEE as substrate) activity in the digestive system of *O. coerulescens* was 1.58±0.16 and 0.612 μmol $min^{-1}mg^{-1}$ protein, respectively. Ortego *et al*. (33) showed that trypsin (BApNA as substrate) and chymotrypsin (SA2PPpNa as substrate) activity in the digestive system of *A. mariaefranciscae* was 92.5±0.6 (at pH 11.5) and 940±5 (at pH 11) nmol $min^{-1}mg^{-1}$ protein, respectively. Also, trypsin K_m and V_{max} values in the alimentary canal of *O. coerulescens* were 0.69±0.01 mM and 560±2 $nmol.min^{-1}mg^{-1}$protein, respectively, when BAPNA was used as substrate (Fig. 5). For BApNA substrate, the K_m value of trypsin serine proteinase in the gut of *Eurygaster integriceps* (*Heteroptera: Scutelleridae*) was 0.6±0.06 mM (34) at pH 9 and 25°C. In this study, the estimated K_m value is similar to the previously reported K_m in other insects (0.08–0.93 mM) (35).

Different classes of enzymes are often found in insect species with different gut pH (4, 36). Insects with alkaline midgut pH typically possess serine proteinases, such as trypsin, chymotrypsin and elastase, which perform optimally at neutral to high pH. Those with more acidic gut conditions (pH 3.5–5.5) – for example, some *Coleoptera* – rely instead on cysteine or aspartic proteinases, which function best at acidic pH (27). However, in the present research, a wide pH range of proteolytic activity against proteinaceous substrates was observed; similar findings were reported for other coleopteran pests (37, 8, 38, 33).

Inhibitor effects on protease activity showed that PMSF, as a serine-protease inhibitor, caused a more significant decrease on proteolytic activity in the gut of *O. coerulescens* than other inhibitors. In addition to PMSF, enzyme activity was noticeably inhibited (67.9%) by using TLCK, which showed that serine proteinases are the major hydrolyzing enzymes in the gut of *O. coerulescens* (Table 1). Digestion of food by serine proteinases is common in lepidopterous insects (26). However, serine proteinases are important for digestion in some coleopteran

species; in coleopterans, proteins are usually digested by both serine and cysteine proteases (8, 39, 40, 2). Oppert *et al*. (30) reported that serine proteinase in the digestive system of *C. angustus* is a dominant enzyme class. Our data are also consistent with the studies of Bian *et al*. (24) on *A. glabripennis*, Al Jabr & Abo-El-Saad (32) on *R. ferrugineus*, and Oppert *et al*. (41) on *L. serricorne*, respectively. Thie & Houseman (42) showed that serine proteinase activity was prominent in the larval anterior midgut of *T. molitor*. Hosseininaveh *et al*. (25) showed the presence of trypsin-like serine proteinases in the midgut of *T. granarium*; no cysteine proteinases were detected in the larval midgut extract, using specific cysteine proteinase substrates, inhibitors and activators. But inhibition assays and zymogram analysis highlighted only small amounts of cysteine proteases in the digestive system of *O. coerulescens*. Examination of the physiological conditions of the alfalfa weevil gut suggested that serine and cysteine proteinases played an important role in weevil digestion (41).

Serine proteases represent one of the main catalytic types of proteases occurring in insects. These classes of proteinases, which belong to a common protein super family, are responsible for the initial digestion of proteins in the gut of higher animals (43). Our results showed that the inhibitory effect of the trypsin inhibitor TLCK on the total protease activity was stronger than that of the chymotrypsin-like protease TPCK; this suggested that trypsin was the predominantly active protease enzyme in the gut of *O. coerulescens* larvae and chymotrypsin was less active. Similarly to our findings, Christeller *et al*. (20) found that the dominant endopeptidase in the midgut of *C. zealandica* was also trypsin. Hosseininaveh *et al.* (25) reported the presence of trypsin-like proteinases in the midgut of *T. granarium* larvae.

The results of SDS-PAGE are in close agreement with those obtained by using inhibition assays. As shown in Fig. 6, PMSF is the strongest inhibitor and zymogram made using azocasein as substrate shows the presence of serine proteinases as the main proteases in the larval gut of *O. coerulescens*. Data resulting from zymogram studies strongly revealed that PMSF and TLCK caused a reduction in band intensity comparatively to control in the gel electrophoresis zymogram.

CONCLUSIONS

In conclusion, we studied the properties of digestive proteinases in *O. coerulescens* larvae to provide a better understanding of the development of biologically-based pesticides. We found that midgut extracts of *O. coerulescens* larvae have azocaseinolytic activity within a broad range of pH values, from acid to alkaline, suggesting that this species has a digestive system based on proteases of different mechanistic classes. The ability of midgut extracts to hydrolyze specific

synthetic substrates (*i.e*., BApNA and BTEE), the elucidation of the pH at which maximal hydrolysis occurs and the extracts sensitivity to protease inhibitors confirmed the presence of trypsin, chymotrypsin, cystein and metalloproteases activities. Only small amounts of chymotrypsin and cysteine proteases are present in the digestive system of *O. coerulescens*, while metalloproteases and trypsin are dominant. Plant proteinase inhibitors may inhibit different classes of insect peptidases, including serine, cysteine and aspartate proteinases, and metallocarboxypeptidases, although most of the proteinase inhibitors known and characterized so far are directed towards serine proteinases (7, 2).

The knowledge gained about protease enzyme classes could be used to evaluate the response of *O. coerulescens* to proteinaceous inhibitors, using metalloprotease and trypsin proteinase classes as digestive enzyme targets.

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