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Suppression of growth and endopeptidases of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) infesting coconut using proteinase inhibitors

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ABSTRACT: Investigations on luminal proteinases of grubs of red palm weevil, *Rhynchophorus ferrugineus* (Olivier) infesting coconut revealed presence of two endodpeptidases *viz*., trypsin (BA*p*NA-ase activity) and elastase-like chymotrypsin (SAAPL*p*NA-ase activity) in all stages of larval development. Highest activity of these proteinases coincided with the active feeding stage (mid-larval stage) of the insect. Aprotinin 50 µg, Soybean Trypsin Inhibitor (SBTI) 50 µg and Phenyl Methyl Sulphonyl Fluoride (PMSF) 1700 µg inhibited trypsin activity of *R. ferrugineus* by 77.4%, 63.1% and 55.9%, respectively. Serine proteinase inhibitors *viz*., aprotinin (50 µg), SBTI (50 µg) and PMSF (1700 µg) had a marginal reduction of elastase - like chymotrypsin activity of *R. ferrugineus* by 32%, 14% and 11%, respectively suggesting the serine nature of the proteinase. *In vivo* bioassay of 250 µM aprotinin on coconut petiole method using early stage grubs of *R. ferrugineus* indicated a significant weight loss of 18.9% due to incorporation of serine proteinase inhibitor, aprotinin in a period of 120 h. Possibility of using serine proteinase inhibitor, aprotinin in the management of *R. ferrugineus* was suggested.

KEY WORDS: *Rhynchophorus ferrugineus,* gut proteinases, proteinase inhibitors, aprotinin, soybean trypsin inhibitor

INTRODUCTION

Among the various insects that affect the coconut production, red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) is the key pest of economic significance. *R. ferrugineus*, a concealed tissue borer, is a lethal pest of palms and is reported to attack 17 palm species world wide. Currently, the pest is reported in 15% of the coconut-growing countries and in nearly 50% of the date palm-growing countries (Faleiro *et al*., 2006). Infested palms, if not detected early and treated, often die. However, palms in the early stages of attack respond to chemical treatment with insecticide. The major components of the Integrated pest Management (IPM) programmed for RPW in coconut are surveillance, maintaining plant and field sanitation, preventive chemical treatment of wounds, filling the leaf axils of young palms with a mixture of insecticide and sand, curative chemical treatment of infested palm, cutting and burning of severely infested palms, trapped adults using food attractants (Rajan and Nair, 1997; Faleiro *et al*., 2006).

It is well established that proteolytic enzymes in insect gut are primarily responsible for the digestion of plants proteins. Since insects are unable to synthesize a number of amino acids, they depend on digestive proteinase and plant proteins to meet

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their nutritional requirements (Bernays and Woodhead, 1984). Proteins are digested in the insects gut by enzymes that are active in fairly alkaline *p*H (Lepidoptera) to slightly acidic *p*H (Coleoptera) and serine proteianses account for 95% digestive activity (Applebaum, 1985). Digestive enzymes such as serine proteases, cysteine proteases and other peptidases excreted into the lumen of the larval midgut are responsible to the food protein digestion (Gatehouse *et al*., 1997) and have been considered as to be potential targets for the insect pest management (Jongsma *et al*., 1995).

Plant-derived proteinase inhibitors (PI) are of a particular interest because they are part of the plant natural defense system against insect predation. Previous studies on the effect of dietary proteinase inhibitor either artificially introduced into defined diets or already present in plant tissues, have shown that these PI can be detrimental to growth and development of a wide range of insects (Ryan, 1990; Hilder *et al*., 1987). Proteinase inhibitors can bind with key digestive proteases of insects feeding on plants, disrupting their digestion and reducing growth and survival (Gatehouse *et al*., 2000). It might be possible to control larval stages of *R. ferrugineus* by identifying potential targets like proteinase inhibitors and also unravel the potential of proteinase-inhibitors from legumes for manipulation in management of RPW.

Disruption of protein digestion by proteinase inhibitors represents an alternative approach to pest management in a world dominated by chemical pesticides which besides increasing the production cost, cause environmental hazards. This approach requires a thorough understanding of the biochemical properties of the proteases from the gut homogenate, characterization of these endopeptidases particularly trypsin (EC 3.4.21.4) and elastase-like chymotrypsin (EC 3.4.21.1) in relation to developmental stages and understanding the way it reacts with classical proteianse inhibitors such as soybean trypsin inhibitor and aprotinin.

Keeping this in view, a study on the endopeptidase activities *viz*., trypsin and elastase-like chymotrypsin) of *R. ferrugineus* grubs and its interaction with proteianse inhibitors has been attempted. In the present study, assay conditions of both the endopeptidases were optimized and the effect of metal ions and inhibitors on trypsin and elastase-like chymotrypsin activity of the crude midgut homogenate from *R. ferrugineus* was determined. Characterization of both endopeptidases in relation to developmental stages was analyzed.

MATERIALS AND METHODS

Insect source: Grubs of *R. ferrugineus* used in this study were collected from infested coconut palms in the Research Farm of ICAR Central Plantation Crops Research Institute (CPCRI), Regional Station, Kayamkulam, Alappuzha district, Kerala located at 9°48' N latitude and 76°19'E longitude at an altitude of 3.05 m above Mean Sea Level. Field strains of *R. ferrugineus* were maintained on succulent coconut crown pieces (cabbage) placed in plastic container at 27 ± 2°C and 70 ± 10% relative humidity that was standardized as optimum rearing condition for the pest. Coconut cabbages were replaced on every alternate day to avoid microbial contamination of the fresh plant substrates used as feeding media. *R. ferrugineus* grubs of various stages *viz*., early-instar (<2 g), mid-instar (2-4 g) and late-instar (>4 g) coinciding the physiological stages of pest were used in the study. Two prominent endopetidases *viz*., trypsin (BA*p*NA-ase activity) and elastase-like chymotrypsin (SAAPL*p*NA-ase activity) were investigated.

Chemical source: Substrate for trypsin-like proteinase N-Benzoyl L-arginine *p*-nitroanilide (BA*p*NA) and elastase-like chymotrypsin Succinyl-ala-ala-pro-leu-*p*- nitroanilide (SAAPL*p*NA) and protease inhibitors such as aprotinin, soybean trypsin inhibitor and phenyl methane sulphonyl fluoride (PMSF) were purchased from Sigma-Aldrich Chemical Company (St. Louis, USA). All other chemicals / reagents obtained from Sisco Research Laboratories, Mumbai were of analytical grade of superior quality. Spectrophotometric measurements were recorded using Cary 50 UV-Visible single

beam spectrophotometer linked to desktop computer.

Preparation of gut extracts: *R. ferrugineus* larvae were sampled two days after head-capsule slippage when the active feeding behaviour of the insect pest was observed. Three different stages of the test insect (early, mid and late-instar) coinciding the physiological stages of development and appropriate age were selected for extraction of gut. Larvae were cold (-20°C) anesthetized for 10 minutes and individual gut was dissected out in insect saline. The dissected gut was isolated free of fat tissues, dehydrated using filter paper, weighed and taken out in Eppendorf tube with 20 mM Tris-HCl, *p*H 8.0. The guts were homogenized using a plastic homogenizer in 1000 µl of 20 mM Tris-HCl, *p*H 8.0. Buffer is added in order to maintain the desired *p*H and thereby maintenance of intact enzyme activity. Homogenates were clarified to remove particulate matter by centrifugation (Hereaus centrifuge) at 12000 rpm for 15 minutes at -4°C. Supernatants were transferred to clean tubes and stored at -20°C for use in peptidase enzyme assay.

Enzyme assay conditions: The trypsin assay condition for the crude gut extract were standardized using 50 mM Sodium citrate buffer *p*H 6.0, 5.0 mM Tris-HCl buffer *p*H 7.0, 8.0, 9.0 and 100 mM Sodium bicarbonate buffer *p*H 10.0, 11.0, temperature ranging from 37-50°C and incubation time ranging from 20-40 minutes using BA*p*NA (1 mM) as substrate. Similarly assay condition for elastase-like chymotrypsin was standardized using buffers ranging from *p*H 6-11, temperature range from 37-55°C and incubation time from 20-50 min using SAAPL*p*NA (1 mM) as substrate. Assays were performed according to Burgess *et al*. (2002) with slight modification in a reaction volume of 4.0 ml comprising of 25 µl of crude gut homogenate, 275 µl water, 100 µl of 1mM BA*p*NA /1mM SAAPL*p*NA, 3200 µl of buffer and 400 µl of stopping reagent. Reaction was started by the addition of 25 µl of crude gut homogenate to the buffered substrate solution and then incubated at relevant temperature. The enzymatic reaction was stopped by the addition 400 µl of 30% acetic acid (stopping reagent) after the required period of incubation as outlined by Josephrajkumar *et al*. (2005). All assays were carried out in duplicate and blanks were used to account for spontaneous breakdown of substrates. Controls were incubated similarly, but acetic acid was added at the beginning of each assay. The peptidase as well as elastase-like chymotrysin activities were determined by the amount of *p*-nitroaniline (*p*NA) released from the substrate and were measured at 405 nm (Thangam and Rajkumar, 2002). The activity was expressed as nanomoles of *p*NA released per minute per gram of the gut tissue (*Molar extinction coefficient of pNA is 9500 M^{-1} cm^{-1})*. Total protein in the crude gut extract was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as standard and expressed as mg g^{-1}. Specific activity was represented as activity per mg protein.

Effect of metal ions on peptidase / elastase-like chymotrypsin activities: In order to determine the optimum metal ion required in enzyme assay, 25 µl of insect gut extract was diluted to 400µl using distilled water and mixed with 3200?l of 50 mM Tris-HCl buffer (*p*H 9.0) in case of peptidase assay and 50 mM Tris-HCl buffer (*p*H 8.0) for elastase-like chymotrypsin consisting of different metal ions $CaCl_2$, $MgSO_4$, $ZnSO_4$, Na_2SO_4, $CuSO_4$ and $HgCl_2$ (20 mM) in separate tubes. After 10 minutes of incubation, 100µl of 1mM BA*p*NA / 1mM SAAPL*p*NA was added and mixed thoroughly. The mixture was incubated at 40°C for 25 minutes and the assays were performed in duplicate as indicated above.

Enzyme activities on different instars of *R. ferrugineus:* In order to determine the enzyme activities in different larval instars, 25 µl of gut extracts of different instars (early, mid and late instars) of *R. ferrugineus* larvae were taken in test tubes and the volume was made up to 400 µl using distilled water. To this 3200µl of 50 mM Tris-HCl buffer (*p*H 9.0) with 20mM Na_2SO_4 was added in case of peptidase assay and 50 mM Tris-HCl buffer (*p*H 8.0) with 20mM $CaCl_2$ for chymotrypsin

assay. The reaction mixture was incubated at 40°C for 25 minutes in water bath after addition of relevant substrate and proceeded as above.

Effect of inhibitors on enzyme activities: Inhibition assays were carried out using aprotinin (0-50 µg), and soybean trypsin inhibitors (0- 50µg) and phenyl methyl sulphonylfluoride (0-1700 µg), which are the classical inhibitors of serine protease. Different amounts of these inhibitors were added to the reaction mixture of 4.0 ml comprising of 25 µl of crude gut homogenate, 175-275 µl water, 100 µl of 1mM BA*p*NA, 3200 µl of 50 mM Tris-HCl buffer (*p*H 9.0) with 20mM Na_2SO_4 and 400 µl of stopping reagent. In case of chymotrypsin assay 50 mM Tris-HCl buffer (*p*H 8.0) with 20mM $CaCl_2$ was used. The reaction mixture was incubated at 40°C for 25 minutes as mentioned in earlier experiments.

Feeding bioassay: Laboratory experiment was conducted at room temperature (28-30°C) in 100 ml plastic cups filled with 40-50 g of fresh skin-pealed coconut petiole. 1 ml of 250 µM of aprotinin was painted on the coconut petiole and fed to seven early larvae of *R. ferrugineus* maintained in separate containers. Similarly control was maintained and larvae fed on coconut petiole devoid of aprotinin. Initial larval weight and weight gain after 120 h of each larvae was recorded. All data were compared with Student's t-test.

RESULTS

Trypsin activity: The trypsin activity on crude midgut homogenates of *R. ferrugineus* was standardized using different *p*H range (6-11), temperature regimes (37-50°C) and incubation time interval (20-40 min). The optimum conditions for trypsin activity with respect to the crude extract of *R. ferrugineus* are 50 mM Tris-HCl (*p*H 9.0) with incubation for 25 min at 40°C.

Among the six metal ions studied, 20 mM concentration of Na_2SO_4, $MgSO_4$, $CaCl_2$, $ZnSO_4$, were found to be stimulatory in that order and exhibited high residual specific activity of more than 50%. Sodium sulphate (Na_2SO_4) was found to be a cofactor for peptidase activity with respect to the crude extract of *R. ferrugineus* (Fig 1).

Results indicated the presence of trypsin activity in all stages of larval development of *R. ferrugineus*. Age related modulation of trypsin activity, protein concentration and specific activity was observed for crude midgut homogenate of *R. ferrugineus* grubs. Trypsin activity was found to be low at early instar (515.3 nanomole *p*NA / min / g), which attained a peak at mid instar (1043.0 nanomole *p*NA / min / g) and further reduced to lowest (288.9 nanomole *p*NA / min / g) at late instar indicating a peak activity at mid-instar of *R. ferrugineus* coinciding the active feeding stage of the insect (Table 1).

A progressive decline in the trypsin activity with increase in the concentration of the serine protease inhibitors *viz*., aprotinin, SBTI and PMSF was observed suggesting the presence of serine residue at active site of the enzyme. Results indicated that 50 µg of aprotinin, 30 µg of SBTI and 1700 µg of PMSF induced inhibitory effect to the tune of 77.4%, 63.1% and 55.9%, respectively, on trypsin activity of *R. ferrugineus*. Aprotinin was found to

Table 1. Trypsin activity on different instars of *R. ferrugineus*

Stage	Activity (nanomole *p*NA / min / g)	Protein (mg /g)	Specific activity (nanomole *p*NA / min / mg protein)
Early-instar	$515.3^{b} \pm 11.1$	$35.3^{b} \pm 2.3$	14.59
Mid-instar	$1043.0^{a} \pm 14.1$	$54.7^{a} \pm 2.9$	19.06
Late instar	$288.9^{c} \pm 7.6$	$27.1^{c} \pm 2.5$	10.66

In columns values followed by same alphabet(s) are not significantly different (P<0.05 DMRT)

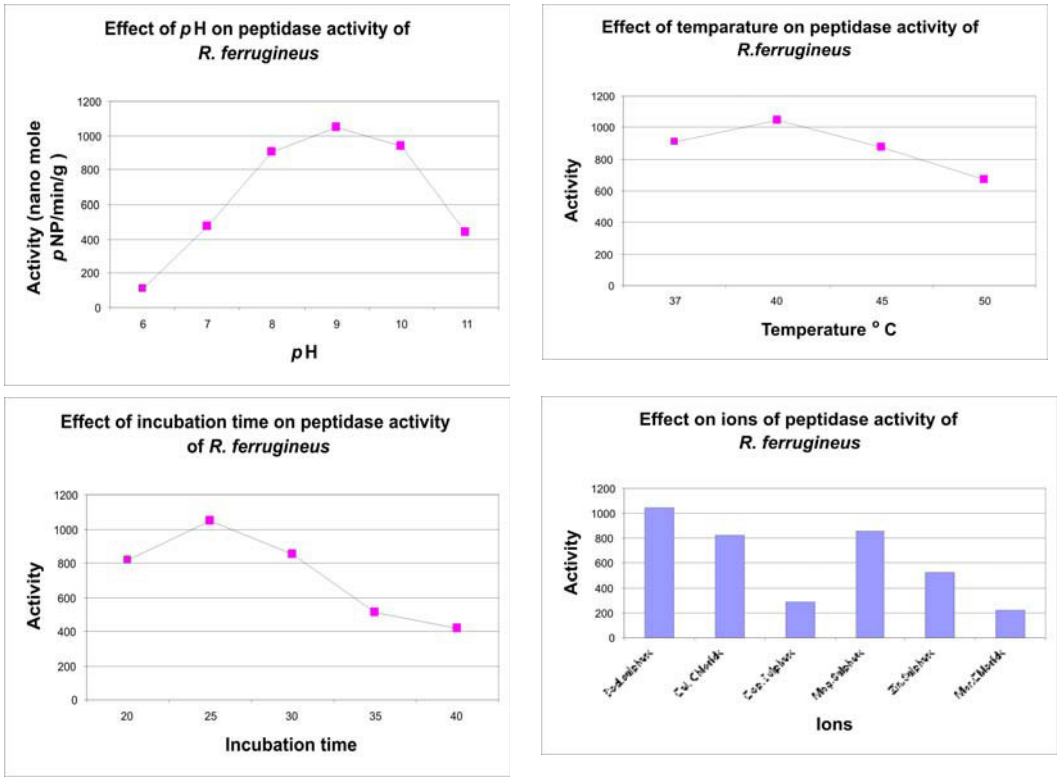

Fig. 1. Standardization of trypsin activity

be more inhibitory than SBTI whereas SBTI was found to be more inhibitory than PMSF for a given concentration of inhibitor on the peptidase activity of *R. ferrugineus* (Table 2). The inhibition pattern of trypsin activity on the crude mid gut homogenate of *R. ferrugineus* was found to be aprotinin > SBTI > PMSF in that order of magnitude.

Elastase-like chymotrypsin activity: The elastase-like chymotrypsin activity (SAAPL*p*NA-ase activity) on crude midgut homogenates of *R. ferrugineus* was standardized using different *p*H range (6-11), temperature regimes (37-55°C) and incubation time interval (20-50 min). Highest activity of elastase-like chymotrypsin in *R. ferrugineus* was recorded at 50 mM Tris-HCl (*p*H 8.0) with incubation for 25 min at 40°C. Among the six metal ions studied, 20 mM concentration of $CaCl_2$ and $MgSO_4$ were found to be stimulatory in that order and exhibited high residual specific activity of more than 60%. Calcium chloride ($CaCl_2$) was found to be a cofactor for elastase-like chymotrypsin activity with respect to the crude extract of *R. ferrugineus* (Fig. 2).

Elastase-like chymotrypsin was also found to be one of the dominant digestive proteinases of *R. ferrugineus* evincing maximum activity (882.6 nmole *p*NA rel/min/g) in mid-instar coinciding the active feeding stage of the insect. Elastase activity was found to be lowest at late-instar (244.3 nmole *p*NA rel/min/g) and comparatively higher at early-instar (354.7 nmole *p*NA rel/min/g) of *R. ferrugineus* (Table 3).

Serine proteinase inhibitors *viz*., aprotinin (50 µg), soybean trypsin inhibitor (30 µg) and phenyl methyl suphonyl fluoride (1700 µg) had a marginal reduction 32%, 14% and 11%, respectively in elastase-like chymotrypsin activity of *R. ferrugineus* suggesting the serine nature of the protease. Among the inhibitors evaluated, inhibition pattern of elastase

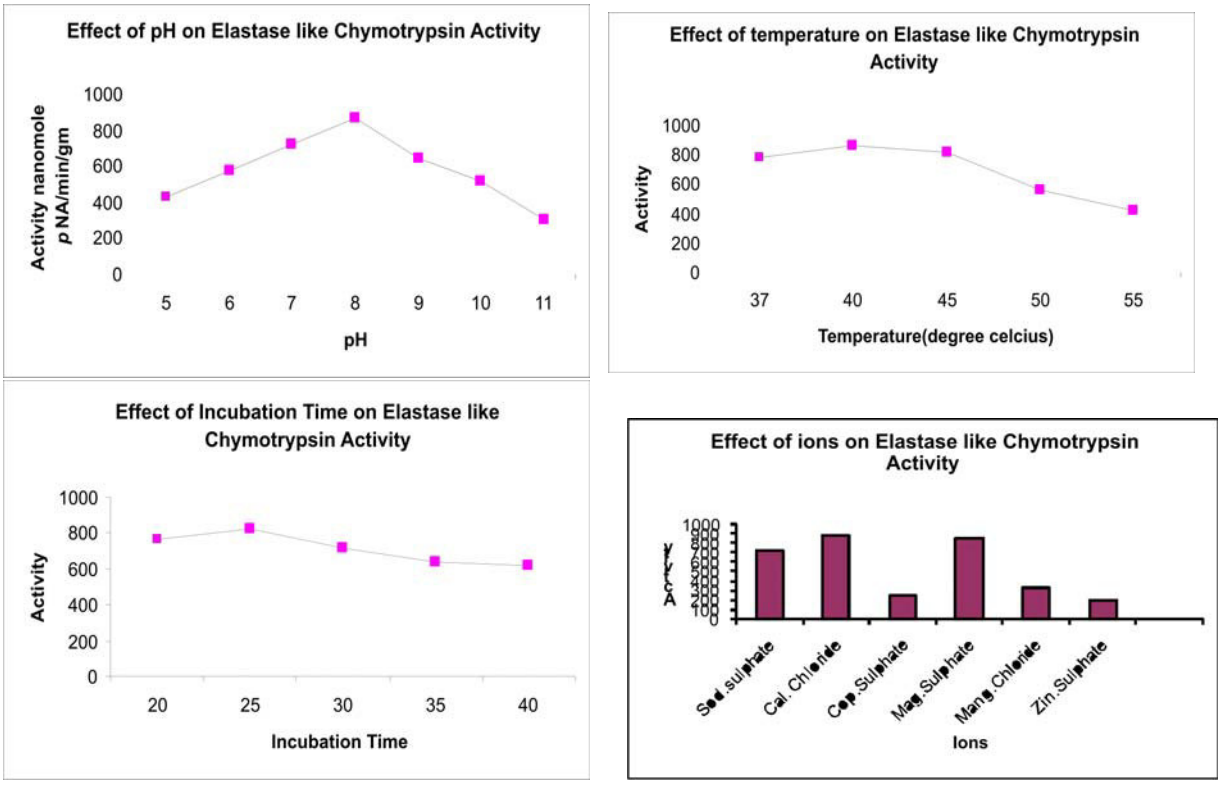

Fig. 2. Standardization of elastase-like chymotrypsin

Table 2. Influence of inhibitors on trypsin activity of *R. ferrugineus*

Aprotinin (?g)	Activity (nanomole *p*NA / min / g)	Soybean trypsin inhibitor (SBTI) (?g)	Activity (nanomole *p*NA / min / g)	Phenyl methyl sulphonyl fluoride (PMSF)	Activity (nanomole *p*NA / min / g) (?g)
0	$1044.8^{d} \pm 14.1$	0	$1044.8^{d} \pm 14.1$	0	$1044.8^{d} \pm 14.1$
10	$652.3^{c} \pm 5.9$	10	$497.3^{c} \pm 9.2$	170	$868.2^{c} \pm 5.9$
25	$295.4^{b} \pm 7.9$	25	$414.9^{b} \pm 4.8$	850	$770.9^{b} \pm 6.8$
50	$235.7^{a} \pm 4.3$	50	$385.6^{a} \pm 8.2$	1700	$459.9^{a} \pm 7.3$

In columns values followed by same alphabet(s) are not significantly different (P<0.05 DMRT)

Table 3. Chymotrypsin activity on different instars of *R. ferrugineus*

Stage	Activity (nanomole *p*NA / min / g)	Protein (mg /g)	Specific activity (nanomole *p*NA / min / mg protein)
Early-instar	$354.7^{b} \pm 13.3$	$36.6^{b} \pm 4.6$	9.7
Mid-instar	$882.6^{a} \pm 16.9$	$54.3^{a} \pm 2.7$	16.3
Late- instar	$244.3^{c} \pm 8.7$	$28.6^{c} \pm 3.2$	8.5

In columns values followed by same alphabet(s) are not significantly different (P<0.05 DMRT)

activity on the crude mid gut homogenate of *R. ferrugineus* was found to be aprotinin > soybean trypsin inhibitor > phenyl methyl suphonyl fluoride in that order of magnitude. The effect of various inhibitors on elastase-like chymotrypsin is presented in table 4.

***In vivo* feeding bioassay:** *In vivo* bioassay of aprotinin (250 mM) on coconut petiole painting method using larvae of *R. ferrugineus* revealed changes in the weight gain of the test insect. In a period of 120 h, the control insect attained 2.305 g weight whereas aprotinin-fed *R. ferrugineus* attained 1.939 g indicating weight loss of 18.9% due to incorporation of serine protease inhibitor.

DISCUSSION

Gut was considered as a vital target for insect control due to its importance in food digestion and nutrient absorption. Dietary protein digestion in insects is initiated by hydrolysis by endopeptidases, followed by carboxypeptidases and aminopeptidases. Endopeptidases degrade the proteins into small peptides, and aminopeptidases and carboxypeptidases further degrade the peptides into amino acids from the amino and carboxyl termini, respectively.

Gaining an insight into the proteolytic properties of the digestive enzymes of *R. ferrugineus* is critical for developing appropriate and effective pest management strategies through protease inhibitors. Results from these studies suggest that protein digestion in *R. ferrugineus* is primarily due to serine proteases that are sensitive to serine protease inhibitors tested. Digestion of food by serine proteases is the preferred mode in lepidopteran insects. Targeting these enzymes may be a good strategy for the development of effective bio-pesticides. Selective inhibition of digestive enzymes in insects induces production of detrimental effects on growth of larvae to prevent digestion and assimilation of nutrients to retard their development and cause their death. Gut homogenates of *R. ferrugineus* in this study displayed substantial enzyme activity only at alkaline *p*H with maximum values recorded at *p*H 9.0 for peptidase activity and *p*H 8.0 for elastase-like activity. This high value is not the same as the optimum *p*H reported for several species of coleopteran insects, which show a neutral (*p*H 7.0) or even slightly acidic (*p*H 5.0) optimum *p*H (Novillo *et al*., 1997). The results obtained in this study on crude gut homogenate of *R. ferrugineus* suggest a major involvement of alkaline proteases in protein digestion. The data strongly suggests the presence of serine proteinases in midgut extracts, confirming the occurrence of protein digestion in the insect.

The trypsin activity as well as chymotrypsin activity of *R. ferrugineus* progressively increased to reach a maximal activity at 40°C and thereafter due to inactivation of the enzyme and linearization of 3D configuration, there was a decline in activity attaining as low as only 59% at 50°C. The trypsin

Table 4. Influence of inhibitors on elastase-like chymotrypsin of *R. ferrugineus*

Aprotinin (µg)	Activity (nanomole *p*NA / min / g)	Soybean trypsin inhibitor (µg)	Activity (nanomole *p*NA / min / g)	Phenyl methyl sulphonyl fluoride (µg)	Activity (nanomole *p*NA / min / g)
0	$842.8^{d} \pm 18.1$	0	$849.8^{d} \pm 15.1$	0	$853.2^{d} \pm 17.1$
10	$830.1^{c} \pm 20.1$	10	$840.2^{c} \pm 17.2$	170	$843.4^{c} \pm 15.0$
25	$713.9^{b} \pm 17.5$	25	$798.2^{b} \pm 14.1$	850	$805.2^{b} \pm 12.1$
50	$573.4^{a} \pm 16.2$	50	$727.4^{a} \pm 15.9$	1700	$760.2^{a} \pm 15.1$

In columns values followed by same alphabet(s) are not significantly different (P<0.05 DMRT)

activity was strongly temperature dependent and was similar to that reported from several other lepidopteran larvae (Bernardi *et al*., 1991).

Some metal ions such as Na^+, Mg^{2+}, Ca^{2+} and Zn^{2+} enhanced BA*p*NA-ase / SAAPLpNA activities whereas others like Cu^{2+} and Hg^{2+} were inhibitory at 20 mM concentration. The specific activities of three ions *viz*., Na^+, Mg^{2+} and Ca^{2+} were found to be above 50 nanomole *p*NA released / min/ mg protein suggesting their possible role as cofactors for trypsin-lke proteases of *R. ferrugineus*. Accumulation of heavy metals due to excessive application of fertilizers and pesticide molecules could possibly alter the trypsin activity of *R. ferrugineus* leading to desensitization and adaptive behaviour. In crude mid gut homogenate of cardamom shoot and capsule borer, *C. punctiferalis* divalent ions such as Ca^{2+}, Mg^{2+}, Pb^{2+} and Co^{2+} exhibited stimulatory effects on peptidase activity, whereas Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Hg^{2+} were inhibitory and the effect of other monovalent ions such as Na^+, K^+, Li^+, Rb^+ and Cs^+ were only marginal (Josephrajkumar *et al*., 2006). When monovalent ion such as Na^+ exhibited marginal effect on lepidopteran insect, *C. punctiferalis* it evinced highest peptidase activity on coleopteran insect, *R. ferrugineus* suggesting the uniqueness of coenzyme for each insect on peptidase activity. Predominance of coconut along sea shore may have contributed the sodium ion as a possible coenzyme for the peptidase activity of *R. ferrugineus* and the possible adaptation of this ion by insect. Absorption of sodium ion by the plant may therefore be well utilized by the insect for its peptidase activity.

During the active feeding stage of the grub the trypsin/chymotrypsin activity was found to be the highest indicating higher consumption of food as well as effective digestion of the food consumed. Food consumption by insects is highly correlated with trypsin/chymotrypsin activity in the gut. A significant amount of inhibitors may have to be ingested during early and active feeding stages of the test insect coinciding with the highest levels of activity of digestive proteinases. There has been a noticeable decrease in the specific activity at the late stage of larval development of *R. ferrugineus* may be coinciding the wandering stage prior to pupation. This decline may result from a greater degradation or a lower synthesis of digestive proteianses produced by a quantitative decrease of the feed intake when larvae is near of the next moult stage or approaching pupation. As the grubs approached pupation, lower levels of proteolytic activity are present in the insect guts, concomitant with decreased feeding activity. Elastase-like chymotrypsin activity was found to be lower than peptidase activity indicating the dominance of trypsin-like proteases in protein digestion of *R. ferrugineus*.

The study demonstrated that, *in vitro*, aprotinin, SBTI and PMSF were effective at retarding trypsin-like (BA*p*NA hydrolyzing) and elastase-like chymotrypsin (SAAPL*p*NA hydrolyzing) activity extracted from the digestive tract of *R. ferrugineus.* It was also found that the inhibition of both endopeptidases on the crude mid gut homogenate of *R. ferrugineus* was found to be aprotinin > SBTI > PMSF in that order of magnitude. As expected, aprotinin was particularly effective at inhibiting both the endopeptidases than the other two inhibitors studied. The results demonstrated a pronounced difference in the sensitiveness of enzyme activities to the inhibitor as the concentration of inhibitors varied for achieving the similar level of inhibition under *in vitro* condition. These results agree to those reported by Oliveira *et al*. (2005) who detected a higher sensitivity of the proteolytic activity of the partially purified fraction to benzamidine than to PMSF.

Besides inhibiting both the endodpeptidases studied, aprotinin significantly suppressed the growth of *R. ferrugineus* indicating effective indigestion of dietary proteins. This is also indicative for effective silencing of these insect specific serine proteinases for retarding growth of *R. ferrugineus*.

The work presents one of the first steps to more precise understanding of biochemical organization of digestive processes in *R. ferrugineus*. Future studies concerning *R. ferrugineus* with particular emphasis on enzyme compartmentalization, substrate specificity and substrate preference as

well as inhibition will deepen our understanding of the digestive processes within this polyphagous Curculionid beetle. Targeting and purification of the these enzymes may be good strategy for the development of effective bio-pesticides and developing transgenics.

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