Plant Cell Wall Degrading Enzymes, Pectinase and Cellulase, in the Digestive System of the Red Palm Weevil, *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae)

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Abstract

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In digestion, the red palm weevil, *Rhynchophorus ferrugineus*, has been adapted to overcome the plant cell wall barrier, specially lignocellulosic and pectic compounds, by producing cellulase and pectinase enzymes. Partial biochemical characterisations of cellulase and pectinase were determined in the larval digestive system of the pest. Larval midgut extract showed an optimum activity for cellulase and pectinase against carboxyl methyl cellulose and pectin at pH 6.0 and 7.0, respectively. Larval midgut cellulase and pectinase were more stable at pH 4.0–8.0 and pH 6.0–8.0 than in highly acidic and alkaline condition, respectively. However, cellulase and pectinase showed to be more stable at pH 6.0 and 7.0, respectively, when the incubation time increased. Maximum activity for cellulase and pectinase incubated at different temperatures was observed at 50°C. Cellulase and pectinase activity significantly decreased in the presence of EDTA and SDS. On the contrary, Ca^{2+}, Mg^{2+}, and Na^{+} significantly affect pectinase activity and K^{+} did not affect the enzyme activities. Ca^{2+} and Mg^{2+} increased cellulase activity as well. K_M and V_{max} for pectinase activity were 0.92 mg/ml and 290 units/mg. Zymogram analyses revealed the presence of one form of pectin methyl esterase and one form of cellulase in the larval digestive system.

Keywords: *Rhynchophorus ferrugineus*; digestive; carbohydrase; midgut; pectin

Abbreviations: CMC – carboxyl methyl cellulose; EDTA – ethylenediaminetetraacetic acid; SDS – sodium dodecyl sulphate; RPW – red palm weevil

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae), is the most important pest of the date palm, *Phoenix dactylifera* (Linnaeus 1753) in the world. It is also an invasive species that is claimed to be originated from South and Southeast Asia, where it is considered as one of the most destructive pests of coconut, *Cocos nucifera* (Linnaeus 1753) (Abraham *et al.* 1975; Sivapragasam *et al.* 1990; Sadakathulla 1991; Murphy & Briscoe 1999; Ferry & Gomez 2002). RPW can eliminate one thousand date trees in Saravan (Iran) annually (Faghih 1996). As the larvae feed, they create frass (chewed up plant fibre) which combines with the plant sap and this fills the tunnels made by the larvae (Murphy & Briscoe 1999). Generally, the damage caused by the larvae can be seen only long after infection, and finally larval damage results in the death of the infected tree. This late detection of the weevil presence constitutes a serious problem in the fight against the pest and in any effort to guarantee the pest-free status in adult trees (Ferry & Gomez 2002). As with most insect pests, control is usually attempted by means of chemical insecticides (Sclar 1994) but it has had serious consequences such as intoxication of people and animals, contamination of water, air, and soil, residues in food, high persistence in the environment, resistance in pests, and impact on beneficial insects, among

other effects (Rodríguez *et al.* 2003; Huignard *et al.* 2005). Because of the concealed nature of the larvae, effective methods for the management of RPW have been difficult to develop (Murphy & Briscoe 1999). Current methods recommended for the management of *Rhynchophorus* species have focused on integrated pest management (IPM) involving surveillance, pheromone lures, cultural control and chemical treatments (Abraham *et al.* 1998). Modification of economically vital crops by the addition of insecticidal proteins is a potentially effective technique to control pests. Genes encoding digestive enzyme inhibitors have been incorporated into plants with differing results (Oppert 2000; Oppert *et al.* 2000). Considerate biochemical characterization of the enzymes active in the midgut of the target pests is the primary step in designing inhibitor-transgenic crops (Oppert 2000; Oppert *et al.* 2000; Wilhite *et al.* 2000).

Pectic substances and cellulose are two important constituents in forming plant cell walls (Maness & Mort 1989). The major component of the middle lamella of the plant cell wall is pectin. Pectin holds plant cells together into a tissue structure. Phytophagous insects, such as RPW, have to utilise some biochemical, mainly pectinase and cellulase, and mechanical tools to overcome the barrier. Numerous pectinases from plants, fungi and bacteria have been purified and characterised (Barash *et al.* 1984; Moshrefi & Luh 1984; Shanley *et al.* 1993). Herbivorous insects utilise their digestive pectinases for degrading the plant cell wall while feeding. Pectin methylesterases, polygalacturonase, pectin lyases and pectate lyases are the main classes of pectinases (Djordjevic *et al.* 1986; Pifferi *et al.* 1989). The roles of pectinases in animals have not been completely understood, but they may play an important role in plant-insect interactions as well. The insects use grouping of these enzymes to degrade the pectin mediated cell wall. Pectinases in aphids, for example, are supposed to be involved in plant penetration and therefore in biotype development and plant resistance (Dreyer & Campbell 1987). Pectinases have been isolated and characterised from two weevils, *Diaprepes abbreviates* and *Sitophilus oryzae* (Doostdar *et al.* 1997; Shen *et al.* 1996) and cloned from a phytophagous beetle, *Phaedon cochleariae* (Chrysomelidae) (Girard & Jouanin 1999).

Cellulose is the major constituent of the primary plant cell wall. Cellulases catalyse the hydrolytic digestion of cellulose and make primary products such as glucose, cellubiose, and cello-oligosaccharides. Insects can use cellulose as a sugar source for energy requirements (Willis 2009). Several insects from 12 orders, such as Dictyoptera, Orthoptera, and Coleoptera may produce their own cellulases (i.e. endo-β-1,4-glucanase) in their midguts and/or salivary glands (Willis 2009; Oppert *et al.* 2010; Watanabe & Tokuda 2010). The term cellulase mainly includes three enzymes leading to glucose production from hydrolysing cellulose (Clarke 1996). The enzymes are endo-β-1,4-glucanases (EG; EC. 3.2.1.4), exo-β-1,4-cellobiohydrolases (CBH; EC. 3.2.1.91), and β-glucosidases (BG; EC. 3.2.1.21). The endo-β-1,4-glucanases and exo-β-1,4-cellobiohydrolases act together to hydrolyse cellulose to small cello-oligosaccharides. The oligosaccharides (mostly cellobiose) are then hydrolysed to glucose by a core β-glucosidase (Sukumaran *et al.* 2005). It seems that insect cellulases are a strong potential to be replaced with the current cellulases used in converting cellulose to glucose in industry (Willis 2009). There has been much information on cellulolytic activity in insects.

The present paper reports some biochemical characteristics of two main polysaccharide degrading enzymes, pectinases and cellulases, in the alimentary canal of *R. ferrugineus*. The resultant information can confidently lead to new strategies for management of the pest.

MATERIAL AND METHODS

Insect. The last larval instars of *R. ferrugineus* were collected from date trees located in the city of Saravan in Sistan & Baluchestan province, Iran, and were used as the source of enzymes in subsequent experiments.

Sample preparation. The last larval instars were cold-immobilised, dissected under a stereoscopic microscope, and their midguts (Figure 1) were re-

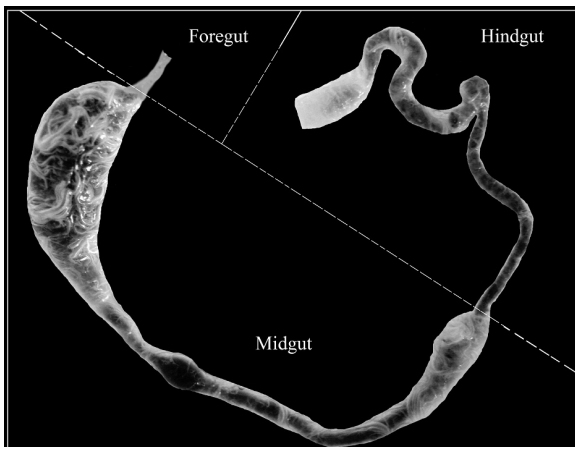

Figure 1. Different parts of the alimentary canal of *R. ferrugineus*

moved. The midguts were then cleaned of unwanted adhering tissues. The midguts, including contents, were collected into a known volume of distilled water and homogenised with a hand-held glass grinder on ice. The homogenates were centrifuged at 15 000 *g* at 4°C for 15 minutes. The resulting supernatants were passed through a filter paper and then were transferred to new tubes and maintained at –20°C for further uses.

Pectinase and cellulase activity assay. A slightly customised colorimetric assay was conducted for detecting pectinase and cellulase activity using 3,5-dinitrosalicylic acid (DNS) as the reagent and 1% soluble pectin and carboxymethyl cellulose (CMC) substrates, respectively. Ten microliters of the enzyme extract was incubated with 85 µl buffer and 15 µl soluble pectin 1% (w/v) (for pectinase assay) or CMC 1% (w/v) (for cellulase assay) for 60 min at 45°C. The reaction was stopped with addition of 60 µl DNS and heating in boiling water for 10 minutes. The absorbance was finally measured at 540 nm. All tests were conducted in triplicates.

pH profile of pectinase and cellulase activity. The effects of pH on the activity of midgut pectinases and cellulases were assayed. The optimum pH for pectinase and cellulase activities was determined using sodium acetate-phosphate-borate buffer at a broad pH range. The assays were performed according to the section "Pectinase and cellulase activity assay". The method and materials of assay for these two enzymes were similar except the substrate.

Effect of temperature on pectinase and cellulase activity. The effect of temperature on the activity of midgut pectinases and cellulases was assayed. A reaction mixture was incubated at different temperatures in water baths for 60 minutes. The assay was performed according to the section "Pectinase and cellulase activity assay".

pH stability of pectinase and cellulose. Stability of pectinases and cellulases was determined at a pH set and two incubation time periods. The enzyme extract was mixed with the buffer and incubated for 1 and 10 h at 37°C. The substrate was then added to the buffered enzyme extract and the enzyme activity was determined as before.

Effect of activators and inhibitors on pectinase and cellulase activities. To experiment the effect of several ions on the enzyme activities, assays were performed in the presence of 5 µl of different concentrations of chloride salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, sodium dodecyl sulphate (SDS), and ethylenediaminetetraacetic acid (EDTA) to the reaction mixture and activity was measured after 60 minutes. The enzyme was pre-incubated with the compounds for 15 minutes.

Kinetic parameters of pectinase. The Michaelis-Menten constant (K_M) and the maximal reaction velocities (V_{max}) of pectinase were determined. The homogenate was incubated in an appropriate buffer (pH 6) at 45°C toward the substrate pectin in concentrations ranging from 0.66 mg/ml to 21.12 mg/ml. The experiments were performed in triplicate. The K_M and V_{max} were evaluated by non-linear regression analysis using the Sigmaplot 10.0 software.

Visualisation of pectinase and cellulase activity. In-gel assays were performed using non-denaturing SDS-PAGE for visualising pectinase and cellulase activities. The enzyme extract was diluted in electrophoresis sample buffer that contained 25% stacking buffer (0.5M Tris-HCl; pH 6.8), 20% glycerol, 2% SDS, and 0.005% (w/v) bromophenol blue. In the case of cellulase, the enzyme sample was partially denatured at 70°C for 3 min for increasing the gel resolution. Following heating, the samples were briefly centrifuged (Oppert *et al.* 2010) and then loaded in 5% stacking and 10% separating polyacrylamide gel. The substrate pectin and CMC were incorporated into the separating gels at a final concentration of 1 and 0.1% for detection of pectinase and cellulase, respectively. After electrophoresis, the gels were washed in citrate buffer (pH 6) containing 2.5% (v/v) Triton X-100 for 45 min and then incubated in citrate buffer (pH 6) for 60 min with gentle agitation. The gels for visualising pectinase were stained with ruthenium red (0.03%) and the bands of pectinase activity appeared as clear areas in the field of the red background of the gel (Mellon & Cotty 2004). In the case of cellulase, the gels were stained with Congo red (0.1%) for 10–15 min at room temperature. The gels were destained by washing in 50 ml of 1M NaCl until cellulase bands became obvious as clear zones where CMC had been ruined due to the enzymatic activity. After 20 min destaining, 100 µl glacial acetic acid was added to the gel for better visualisation (Waeonukul *et al*. 2007; Willis *et al*. 2010).

RESULTS

Pectinase activity. Pectinase activity, toward the substrate pectin, was observed in the larval midgut of *R. ferrugineus.* The enzyme was optimally active at neutral pH (7.0) (Figure 2a). Lower activity was observed at pH 3.0–6.0 and 8.0–11 and no activity was determined at pH 2.0. Pectinase was active at

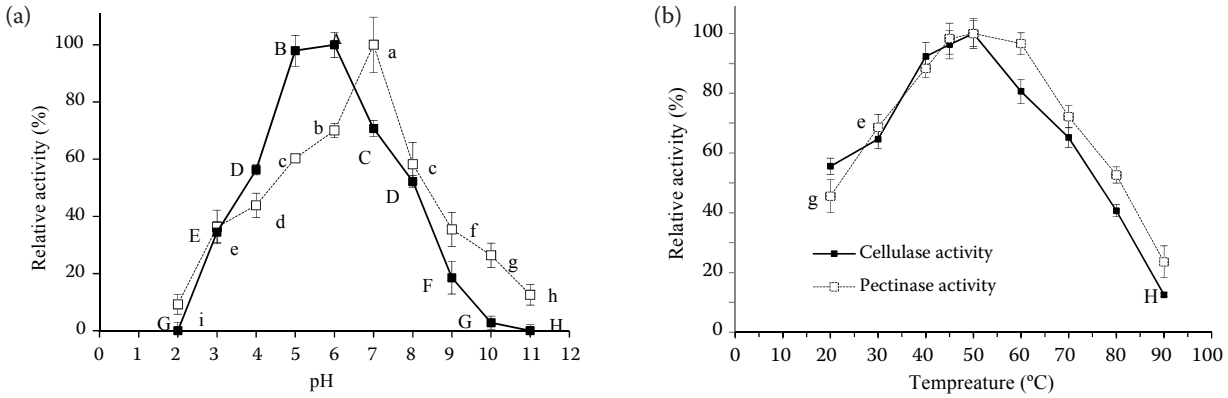

Figure 2. Effect of different (a) pH and (b) temperature range on pectinase from the midgut of *R. ferrugineus*

least 80% of its maximal activity at a broad temperature range, 40–60°C, with maximum activity at 45–50°C (Figure 2b). The enzyme was active at a high temperature (70–80°C) near to 50% of its maximal activity. No activity was determined at 90°C due to the protein denaturing. Results revealed that pectinase was stable (more than 60%) at slightly acidic (pH 6.0) to neutral condition (pH 7.0) in a short and long incubation time (Figure 3). However, the stability of pectinase decreased in a long incubation time (10 h) compared with a short incubation time in the acidic condition. The highest stability of the enzyme was determined at pH 7.0. Increased concentrations of Mg^{2+}, Na^{+}, and Ca^{2+} enhanced pectinase activity (Figure 3). EDTA and SDS showed an inhibitory effect on pectinase activity. K^{+} did not show any significant effect on the enzyme activity. The values K_M and V_{max} for pectinase activity were 0.92 mM and 290 units/mg, respectively. The zymogram analysis showed one band of pectin methyl esterase in the larval midgut of *R. ferrugineus* (Figure 4).

Cellulase activity. Optimum pH for cellulase activity from the larval midgut of *R. ferrugineus* was obtained at pH 5.0 and 6.0 (Figure 2a). The enzyme activity was gradually enhanced with increased pH from 2.0 to 5.0 and decreased in alkaline condition from pH 6.0 to 11.0. No cellulase activity was observed at pH 2, 10, and 11. The digestive cellulase was active (above 30% of its maximal activity) at a broad temperature range from 20°C to 80°C (Figure 2b). Optimum temperature for cellulase activity was obtained at 50°C, however, the enzyme is remarkably active at 40 and 45°C (more than 90%). Cellulase activity gradually decreased at temperatures above 50°C. The enzyme retained its activity at a broad pH range of 4.0–8.0 (Figure 3). However, cellulase was more stable at pHs 5.0 and 6.0 than in acidic, neutral, and alkaline condition. There was no obvious decrease in cellulase activity after 10 h compared with 1 h incubation time.

The effect of some chemicals on cellulase activity was examined. Among the ions, Ca^{2+} and Mg^{2+} significantly increased cellulase activity (Figure 4). The ions K^{+} and Na^{+} did not significantly affect cellulase activity in the midgut of the pest. SDS and EDTA decreased cellulase activity in increased concentrations. The zymogram analysis revealed the presence of one band of cellulase activity in the larval midgut of *R. ferrugineus* (Figure 5).

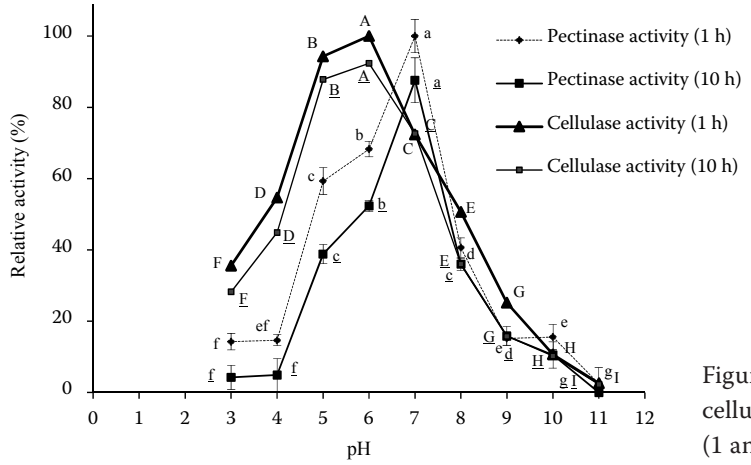

Figure 3. The stability of the midgut pectinase and cellulase at different pHs and two incubation periods (1 and 10 h) in *R. ferrugineus*

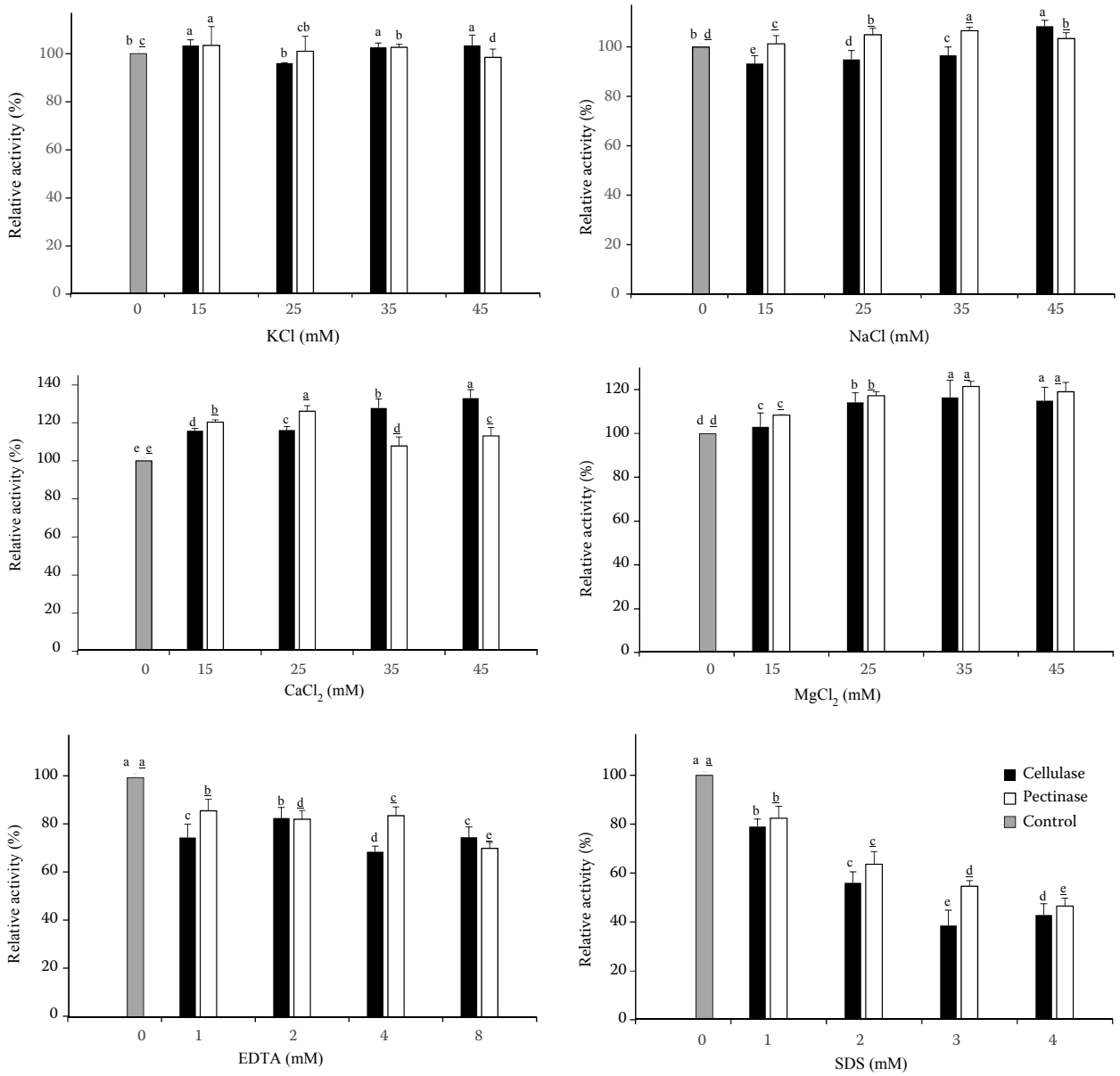

Figure 4. Effect of some ions on pectinase and cellulase activity of the larval midgut extract from *R. ferrugineus*. Mean values (n = 3) with different lowercase letters shows significant differences ($P < 0.05$)

DISCUSSION

The paper presented a comprehensive study of pectinase and cellulase activity in the digestive system of the red palm weevil. Pectin is the main cell wall part of plants and could be used as an energy source for insects. Pectinases are responsible for degrading pectin and exist in the orders Orthoptera, Coleoptera, Hemiptera, Diptera and Tricoptera, even though they are not apparently present in Dictyoptera and in some Orthoptera and Coleoptera (Vonk & Western 1984). *R. ferrugineus* larvae are mainly internal feeders of most parts of the upper trunk where they need to utilise plant cell wall degrading enzymes for digestion. Our results revealed the presence of pectinases in the larval midgut of *R. ferrugineus*. Optimum pectinase activity occurred at neutral pH (7.0) that is nearly consistent with the pH prevailing in the herbivorous coleopteran larval midgut. Acidic optimum pH for pectinase activity was previously reported in some insects. The rice weevil, *Sitophilus oryzae*, feeds on the seeds of cereals, mainly wheat and rice, which contain pectin and it seems likely that the pectinases in the rice weevil are employed as digestive enzymes. In rice weevil, the enzyme is active from pH 4.5 to 6.5 (Shen

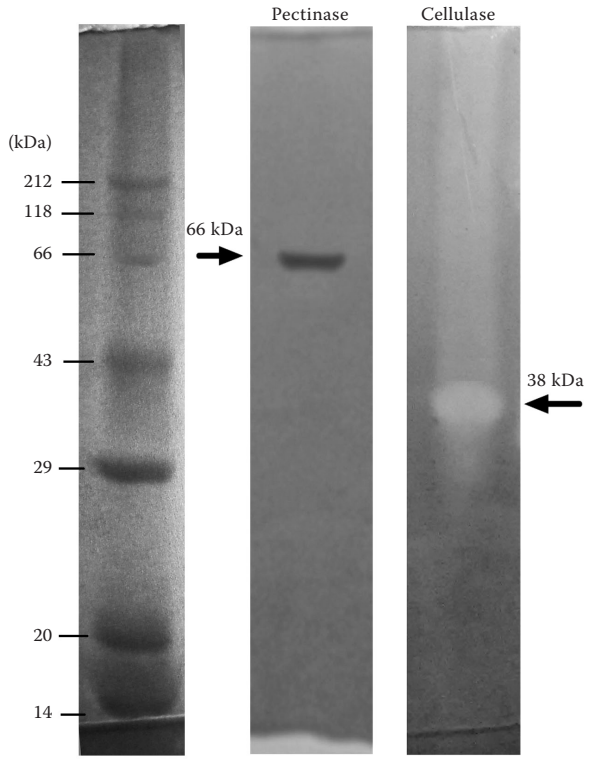

Figure 5. Protein molecular marker (left), zymogram analysis of the midgut pectinase (middle) and cellulase (right) from *R. ferrugineus*

et al. 1996). However, pectin methyl esterases are active throughout the pH range of 4–9, with a peak at pH 8 in *Anthonomus grandis*. Pectin was obviously tainted with a mixture of pectin methyl lyases and polymethyl galacturonases, below pH 6.5 (King 1972). The pectinase zymogram analysis revealed the presence of one form of pectin methyl esterase active in the larval midgut of *R. ferrugineus*. Pectinases in *R. ferrugineus* larvae can be produced either by its own larval digestive system or microorganisms harboured in the alimentary canal of the pest. In *R. ferrugineus*, pectinase activity is enhanced in the presence of the ions Ca^{2+}, Mg^{2+}, and Na^{+}. Increased concentrations of $CaCl_2$ and $MgCl_2$ reduced the quantity of pectinase activity from tomato to about 50% of maximum levels with consecutive extractions (Jackman *et al.* 1995). Requirement for Ca^{2+} and specificity for de-esterified substrates, pollen polygalacturonase is the most effectual in hydrolysing relatively large galacturonans (Pressey & Reger 1989). The ions Hg^{2+}, Mn^{2+}, and Zn^{2+} inhibited 100, 75, and 50%, respectively, of the pectinase activity from the fungus *Thermoascus aurantiacus* (Martins *et al.* 2007). $CaCl_2$ (5mM) improved the activity of pectinase in *Penicillium chrysogenum* by 3.56%. The salts $MgCl_2$ and $ZnCl_2$ inhibited pectinase activity to the level of 21.2 and 14.8%, respectively. Similarly $HgCl_2$, $CoCl_2$, and $CuSO_4$ have been reported to inhibit the pectinase activity of *P. chrysogenum* up to 60%. The compounds $BaCl_2$ and EDTA exhibited maximum inhibition of 40% on pectinase activity (Banu *et al.* 2010). Literature reviews indicate varying amounts of kinetic parameters for the pectinase enzyme. However, in the closest one to the present study, the K_M value for *R. ferrugineus* pectinase is different from and smaller than the K_M value for polygalacturonase in *S. oryzae* (Shen *et al.* 1996), which can be due to the nature of the enzymes. Our study shows that there is one form of pectinase activity in the larval midgut extract of *R. ferrugineus*. It seems that the only band of pectinase activity is more likely to be related to the pectin methyl esterase (PEs) activity because of producing a dark band in the red background in zymogram analyses. In a review study of microbial pectinolytic enzymes, the range of molecular weights in 35–50 kDa was reported for most pectin methyl esterases. PEs are active at a range of pH from 4.0 to 8.0. Fungal PEs have a lower pH optimum than those of bacterial origin. The majority of PEs need for maximal activity an optimum temperature range from 40°C to 50°C (Assis *et al.* 2004). Insects have evolved efficient strategies to use lignocellulosic substrates as sources of energy, which makes them an optimal resource for novel cellulolytic enzymes (Cleveland 1924; Oppert *et al.* 2010; Watanabe & Tokuda 2010). The insect digestive system exhibits states that are optimised for degradation of plant biomass, as well as reducing conditions and high alkaline pH in a number of insect orders (Lehane & Billingsley 1996). The plant cell wall is typically composed of cellulose situated between the middle lamella and the plasma membrane. Cellulose is the major abundant organic polymer (Sukumaran *et al.* 2005) and is degraded by the catalysing action of cellulase in the insect digestive system (Oppert *et al.* 2010; Watanabe & Tokuda 2010). In spite of the presence of many carbohydrolytic activities in insects, their cellulolytic mechanisms are weakly understood. While cellulase genes are not present in the genomes of *Drosophila melanogaster* (Diptera: Drosophilidae) or silk worm *Bombyx mori* (Lepidoptera: Bombycidae), other insects such as termites produce their own cellulases (Watanabe & Tokuda 2010). In the present study, cellulase activity in the larval stage of *R. ferrugineus* was discussed. Our results of pH activity showed that optimum cellulase activity in the larval midgut fluid occurs at acidic condition. Optimal pH of the major endo-β-1,4-glucanase component from *R. ferrugineus*

is similar to that of *Nasutitermes takasagoensis* (Isoptera: Termitidae) (Tokuda *et al.* 1997). In the studies on coleopteran insects, optimum endoglucanase activity was observed at acidic pH (4.8) and 40°C in *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Rehman *et al*. 2011). Endo-β-1,4-glucanase activity for larval stage midgut crude protein occurs in the pH range of 6.2 to 6.9 in *Monochamus marmorator* Kirby (Coleoptera: Cerambycidae) (Kukor & Martin 1986). Cellulase (endo-β-1,4-glucanase) activity in *Apriona germari* (Coleoptera: Cerambycidae) and *Anoplophora glabripennis* (Coleoptera: Cerambycidae) was optimum at pH 4.4 and 5.2, respectively (Yin *et al.* 2000; Li *et al.* 2008). The studies on the isolation of cellulase from beetles demonstrated that optimum pH for the highest activity of cellulase from the larval gut of *Psacothea hilaris* against CMC was 5.5 (Sugimura *et al.* 2003). The results showed that $CaCl_2$ and $MgCl_2$ increased significantly endo-β-1,4-glucanase activity in *R. ferrugineus*. Unfortunately, we could not find any appropriate reports on the impacts of the ions on endo-β-1,4-glucanase activity in insects. The molecular weight in a comparison of enzymatic properties among purified endo-β-1,4-glucanases of higher termites (*Nasutitermes takasagoensis* and *Macrotermes mulleri*), lower termites (*Reticulitermes speratus*), cockroach (*Panesthia cribrata*), and longicorn beetle (*Ergates faber*) was between 25 kDa and 53.6 kDa. The range of optimum pH for this study was 4–6 for this enzyme. The range of optimum temperature and stability temperature of this enzyme was 37–65 and 42–60°C, respectively (Tokuda *et al*. 1997). Our study shows that there is one form of cellulase activity in the larval midgut extract. Cellulase and pectinase activities exist in the gut extract of the red palm weevil. Certainly, these enzymes play an important role in the digestion of the insect. Insect-resistant crops have been among the main achievements of applying plant genetic engineering skills to agriculture (Sharifi *et al.* 2011). The secondary metabolites in plants can take action as defensive agents against insects either by repellence or during direct toxicity. Many dissimilar types of secondary metabolites, as well as alkaloids, terpenes, steroids, iridoid glycosides, aliphatic molecules, phenolics (Hsiao 1985) and others, have been established to confer resistance to different plant species against insects. Among them, carbohydrase inhibitors seem to play an important role in host plant resistance to insects (Sharifi *et al*. 2011). Study of the carbohydrates in insects is vital not only for thoughtful digestion biochemistry but also for rising insect pest management strategies.

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