Biochemical characterisation of digestive \(\beta\)-amylase of Red Palm Weevil, Rhynchophorus ferrugineus (Olivier, 1790) (Coleoptera: Curculionidae)

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Biochemical characterisation of digestive α-amylase of Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier, 1790) (Coleoptera: Curculionidae)

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The Red Palm Weevil, *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae), is a serious pest of a wide range of plant species including coconut, sago, date and oil palms. The α-amylases are the hydrolytic enzymes that are involved in carbohydrate metabolism in insects. So far nothing is done to demonstrate α-amylase activity of *R. ferrugineus*. Thus, the aim of the current study was to identify and characterise the α-amylase activity to gain a better understanding of digestive physiology of the insect. Thus, the α-amylase in the gut of red palm weevil was isolated and characterised using starch as a substrate. The study showed that the α-amylase is present in the gut of the insect for carbohydrate digestion. The α-amylase has an optimum pH and temperature of 5 and 40°C. The activity of α-amylase was increased by NaCl and KCl and inhibited by other compounds such as MgCl\textsubscript{2}, CaCl\textsubscript{2}, urea, ethylenediaminetetraacetic acid and sodium dodecylsulfate. Native-PAGE electrophoresis of α-amylase showed two isoenzymes, one major and one minor band showing α-amylase importance in the carbohydrate metabolism of the insect. Understanding of the digestive physiology and α-amylase activity of Red Palm Weevil is important when new management strategies for this economically important pest are devised.

**Keywords:** α-amylase; *Rhynchophorus ferrugineus*; native-PAGE; inhibitors; temperature

Introduction

The Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae), is a cosmopolitan and multivoltin insect (up to three generation per year) that causes serious damage to a wide range of plant species including coconut, sago, date and oil palms. It is native to Middle East countries where it is called Asian palm Weevil, and it has been widely distributed in Southern Asia (Rajamanickam et al. 1995). It has been reported from Iran since 1992 and has been speculated that this pest crossed into Iran from Pakistan (Murphy and Briscoe 1999). Females lay
their eggs inside the wounds, cracks and crevices or into the hole made by them. After hatching larvae feed on the surrounding palm tissue and bore their way into the centre of the palm. Larvae have three to five instars which last for about two months after which pupal stage is emerged. Pupal stage also may last about two to six weeks and then adults are emerged. Thus, entire life cycle may take about two to four months depend on environmental and feeding conditions (Avand-Faghih 1996).

Control measures are needed in area where high infestation occurs and the most common control tool used against this pest is chemical control which applied in different ways, including dusts, liquid sprays, trunk injection or even soil application of systemic insecticides (Abraham et al. 1998). However, pesticide use has its own limitation because it causes the insects to develop resistance against insecticide as a result the insecticide will no longer affect the insect. Moreover, in addition to the high cost, they pose risk to the balance of the nature, foodstuffs and human health, water quality, wildlife, and the environment as a whole. Thus, the new way of control is needed to diminish reliance on insecticides. Host plant resistance can reduce damage of R. ferrugineus because the insect is unable to exploit hosts effectively. Insect-resistant transgenic crops are promising because commercial release of first-generation maize and cotton expressing single-modified Bacillus turingiensis toxin have been successful (Christou et al. 2006; Ferry et al. 2006; Allahyari et al. 2010).

Many insect species including R. ferrugineus which lives on a carbohydrate-rich diet and thus are dependent on the carbohydrases for their survival. The study of insect digestive enzymes seems to make sense since the insect gut is the major interface between the insect and its environment. Hence, an understanding of digestive enzyme function is essential when developing methods of insect control such as the use of enzyme inhibitors and transgenic plants to control insect pests (Bandani et al. 2001; Ghoshal et al. 2001; Maqbool et al. 2001). Using inhibitors to insect digestive enzyme have already been demonstrated to be an important biotechnology system in the control of insect pest. A major aspect in the pest control is to achieve the selective inhibition of the digestive enzyme/s. Thus, causing detrimental effects on the insect growth and development by preventing digestion and assimilation of nutrients (Confalonieri 1988). The α-amylase (α-1,4-glucan-4-gluconohydrolases; EC3.2.1.1) are the hydrolytic enzymes that are found in microorganisms, plants and animals. This enzyme catalyses the hydrolysis of α-D-(1,4)-glucan linkage in starch and related carbohydrates (Strobl et al. 1998). α-Amylase activity has been described from different species of several insect orders including Coleoptera, Hymenoptera, Diptera, Lepidoptera and Hemiptera (Baker and Woo 1985; Terra et al. 1988; Mendiola-Olaya et al. 2000; Oliveira-Neto et al. 2003; Kazzazi et al. 2005).

They are the important enzymes involved in digestion and carbohydrate metabolism in insects (Horie 1975). Inhibitors of α-amylase inhibitors have already been used in the control of insect pest. Pea and azuki transgenic plants expressing α-amylase inhibitors from common beans (α-AI) were completely resistant to the Bruchus pisorum and Callosobruchus chinensis weevils (Morton et al. 2000; Carlini and Grossi-de-Sa 2002; Franco et al. 2002; Svensson et al. 2004). Barbosa et al. (2010) showed up to 88% inhibitory effect of Coffea arabica transgenic seed extracts against Hypotheneumus hampei α-amylase. Dias et al. (2010) demonstrated that rye α-amylase inhibitor expressed in transgenic tobacco seeds (Nicotiana tabacum) caused 74% mortality in Anthonomus grandis first larval instar when transgenic seed flour mixture was used in artificial diet. An understanding of how digestive enzymes function is essential when developing methods of insect control such as the use of
enzyme inhibitors and transgenic plants to control phytophagous insect species. For nearly all of these strategies, it is important to have a strong understanding of the target insect pest. An understanding of biochemistry and physiology of feeding adaptation is also important. So far, no studies have been done to demonstrate \( \alpha \)-amylase activity of *R. ferrugineus*. Thus, the aim of the current study was to identify and characterise \( \alpha \)-amylase activity to gain a better understanding of digestive physiology of the *R. ferrugineus*. The knowledge thus obtained hopefully will lead to new management strategies for this economically important pest.

Materials and methods

Insect collection and rearing

Last instar Larvae were collected from the date fields around Saravan (Sistan and Baluchestan province) and transferred to the laboratory in Plant Protection Organisation. The insects were kept at laboratory condition on date trunk and branches.

Sample preparation and enzyme assays

Sample preparation was done as described by Allahyari et al. (2010) with slight modifications. Briefly, larvae were placed on ice (about 5 min) for immobilisation and dissected under light microscope. Larval gut was removed and homogenised in pre-cooled homogeniser (Teflon pestle with 0.1 mm clearance) in distilled water. Samples were then put in the 1.5 ml centrifuge tubes and centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was separated and kept at -20°C for subsequent analysis as an enzyme source.

\( \alpha \)-Amylase activity determination

\( \alpha \)-Amylase activity was assayed by the dinitrosalicilic acid procedure (Bernfeld 1955), using 1% soluble starch (Merck, Product Number 1257, Darmstadt, Germany) solution as substrate as described by Bandani et al. (2009). One unit of \( \alpha \)-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35°C. A standard curve of absorbance against amount of maltose released was constructed to enable calculation of the amount of maltose released during \( \alpha \)-amylase assay. Serial dilutions of maltose (Merck, Product Number 105911, Mr 360.32 mg/M) in the Universal buffer (pH 6.5) were made to construct standard curve. A blank without substrate but with \( \alpha \)-amylase extract and a control containing no \( \alpha \)-amylase extract with substrate were run simultaneously with reaction mixture. All assays were performed in triplicates and with three time repetitions.

Gut pH determination

To have a clear understanding of the process of digestion in *R. ferrugineus* and to determine the alimentary canal pH, the last instar insects were dissected under light microscope and their gut removed. The gut pH was determined according to the methods of Bignell and Anderson (1980). Each section of the gut was cut and mounted on a microscope slide, then 5 ml of pH indicator solutions was added to each. Indicators used were: 0.1% bromophenol blue (pH 3.0–4.6), 0.1% methyl red (pH 4.4–6.2), 0.1% brom cresol purple (pH 5.2–6.8), 0.1% bromophenol blue (pH
6.2–7.6), 0.1% natural red (pH 6.8–8.0), 0.1% cresol red (pH 7.2–8.8), 0.1% thymol blue (pH 8.0–9.6) and 0.1% Alizarin yellow (pH 10–12).

Effect of PH and temperature on \( \alpha \)-amylase activity

The effects of temperature and pH on \( \alpha \)-amylase activity were examined using enzyme extract from the larval gut as described by Kazzazi et al. (2005). The optimal pH was determined using universal buffer with pH set at 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The effect of temperature on \( \alpha \)-amylase activity was determined by pre-incubating of the reaction mixture at 10, 20, 25, 30, 35, 40, 50 and 60°C for 30 min followed by measurement of activity as described before.

Effect of activators and inhibitors on \( \alpha \)-amylase activity

To test the effect of different ions on the enzyme activity, assays were performed in the presence of different concentrations of chloride salts of NaCl (5, 10, 20 and 40 mM), KCl (5, 10, 20 and 40 mM), CaCl\(_2\) (5, 10, 20 and 40 mM), MgCl\(_2\) (5, 10, 20 and 40 mM), and ethylenediaminetetraacetic acid (EDTA; 0.5, 1, 2 and 4 mM), sodium dodecylsulfate (SDS; 2, 4, 8 and 16 mM) and urea (2, 4 and 8 M) as described by Kazzazi et al. (2005). These compounds were added to the assay mixture, then incubated at 40°C for 30 min, and the absorbance was read as described before. A suitable blank was also included.

Native PAGE

The amylase present in crude homogenates of the gut after SDS–polyacrylamide gel electrophoresis (PAGE) was visualised using the procedure described by Laemmli (1970) and Campos et al. (1989), with slight modification. SDS–PAGE was performed in a 10% (w/v) separating gel and a 5% stacking gel, both with 0.05% SDS. The electrode buffer was prepared based on Laemmli (1970), but SDS was not used. The sample buffer contained 25% stacking buffer (0.5 mol/l Tris–HCl [pH 6.8]), 20% glycerol, 2% SDS, 0.005% (w/v) bromophenol blue, but with no mercaptoethanol, and it was not heated. Electrophoresis was conducted at 4°C at 100 V until the blue dye reached the bottom of the slab gel. To prepare gels for \( \alpha \)-amylase assay, the gel was cleaned with water and washed by shaking gently with 1% (v/v) Triton X-100 in phosphate buffer containing 2 mM CaCl\(_2\) and 10 mM NaCl for 1.5 h. Staining the gel with 0.05% KI and 0.05% I\(_2\) solution visualised \( \alpha \)-amylase activity as light bands in dark background.

Protein determination

Protein concentration was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, München, Germany) as a standard.

Results

\( \alpha \)-Amylase activity

Studies showed that \( \alpha \)-amylase activity is present in the gut of \textit{R. ferrugineus} larvae and the activity of midgut enzyme was 0.043 U (U/ml). These results showed that
the larvae use α-amylase for digestion and absorption of starch present in the date palm.

**Gut luminal pH**

Red Palm Weevil alimentary canal consists of a short foregut, a massive midgut and a long hindgut (Figure 1). Application of pH indicators showed that foregut was acidic (pH 5.0–5.5), the midgut was less acidic (pH 5.2–6.0) and the hindgut is acidic (pH 5.0–5.8).

**Effect of pH and temperature on α-amylase activity**

Similar to most weevils α-amylases, the optimal pH of red palm weevil α-amylase extracted from the guts was 5.0 (Figure 2). The enzyme activity gradually increased from pH 3.0 to 5.0 and then decreased (Figure 2). However, at pH 4.0, 5.0, 6.0 and 7.0, α-amylases activity did not change significantly. A sharp decrease in α-amylases activity was seen at pH 8.0 that at this pH, about 80% of activity was lost showing that the enzyme is more sensitive to alkaline pH. α-Amylase was active over a broad range of temperatures from 30 to 50°C. However, the highest activity was seen at 40°C (Figure 3). There was a sharp decrease in enzyme activity after 60°C. In other words, at 70°C, the enzyme was completely denatured and its activity was equal to zero. At the other extreme, there was significant difference in the activity of the enzyme between 20 and 30°C.

Figure 1. Alimentary canal of *Rhynchophorus ferrugineus* (Coleoptera: Curculionidae). Red Palm Weevil alimentary canal consists of a very short foregut, a massive midgut and a long hindgut.
Effect of activators and inhibitors on enzyme activity

Among different ions tested, only NaCl and KCl increased the amylase activity (Table 1). The highest activity was obtained when 20 mM of both ions (NaCl and KCl) were used. Ca$^{2+}$ and Mg$^{2+}$ ions did not activate the enzyme activity. In both these ions, the inhibitory activity was increased with increasing ion concentration (Table 1).

Three other compounds tested including EDTA, SDS, and urea had inhibitory effect on the insect amylase activity.

Zymogram analysis of α-amylase activity

Zymogram analysis of the midgut α-amylase revealed two bands of amylase activity (Figure 4) with one major band and one minor band. Mobility relative to that of bromophenol blue for midgut bands was 0.66 (major band) and 0.8 (minor band).
Discussion

The present study for the first time showed that $\alpha$-amylase is present in the digestive system of the *R. ferrugineus*. $\alpha$-Amylases have been found to be active in different insect species (Takagi et al. 1971; Baker 1991; Kazzazi et al. 2005; Zibaee et al. 2008).

Red palm weevil gut pH was determined to be acidic (pH around 5) which is in congruent with the other insects (especially weevils) reports. It has been reported that insect $\alpha$-amylases are generally more active in neutral to acidic pH conditions (Terra and Ferreira 2005). In many Coleopteran insects including *Callosobruchus maculatus*, *Rhizopertha dominica*, *Sitophilus granarium*, *Prostephanus truncatus*, *Tribolium confusum*, *T. castaneum* and *Dermestes maculatus*, optimal $\alpha$-amylases pH has been reported to be acidic (pH 4.0–6.0) (Caldeira et al. 2003; Mehrabadi et al. 2011). However, some members of Dermestidae family, such as *Attagenus megatoma* have alkaline pH (Johnson and Rabosky 2000). In Hemipteran insects, such as true bugs, $\alpha$-amylases optimal pH was slightly acidic, too (Zeng and Cohen 2000; Bandani et al. 2009). Generally, optimal pH is corresponding to the pH prevailing in the midgut from which the enzyme has been extracted so these discrepancies seen in the midgut pH are related to the different feeding habits and feeding sources.

Table 1. Effect of different compounds on the activity of $\alpha$-amylase in *Rhynchophorus ferrugineus* larvae.

<table>
<thead>
<tr>
<th>Component</th>
<th>Density (mM)</th>
<th>Percentage activity</th>
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<tbody>
<tr>
<td>Blank NaCl</td>
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<td>CaCl$_2$</td>
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<td>101</td>
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<td>MgCl$_2$</td>
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<td>EDTA</td>
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<td>Urea</td>
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The optimal temperature for Red Palm Weevil $\alpha$-amylase activity was 40°C, the enzyme was active over a broad temperature range from 20 to 60°C. In the other coleopteran order, enzyme activity had shown to be at 35°C in Cerambyx cerdo L. (Cerambycidae) (Applebaum 1985) and 25°C in Tenebrio molitor L. (Tenebrionidae) (Pereira et al. 1999). In the current study, Mg$^{2+}$ and Ca$^{2+}$ did not activate the $\alpha$-amylase of the Red Palm Weevil. The same is true for the $\alpha$-amylase of the other insects including the Sunn pest, Eurygaster integriceps (Hemiptera: Scutelleridae) (Kazzazi et al. 2005). Also, it has been reported that in Bacteria, genus Thermus, Ca$^{2+}$ did not activate $\alpha$-amylase (Shaw et al. 1995). There are some reports that some $\alpha$-amylases are metalloproteins which require calcium for their optimum activity. In some other organisms, $\alpha$-amylase needs calcium ion for their stability against extreme pH and temperatures (Baker 1983). Red Palm Weevil $\alpha$-amylase has the same feature as the other insect $\alpha$-amylases such as sensitivity to Chelating agent (EDTA), which absorb metal ions, urea and SDS (Terra and Ferreira 2005).

It was found that Red Palm Weevil has a mixture of two different $\alpha$-amylase isoenzymes with different relative mobilities to that of bromophenol blue. A mixture of different $\alpha$-amylase isoenzymes has been reported for other insects such as Sitophilus oryzae, T. castaneum, Anthonomus grandis, C. maculatus, R. dominica, S. granarius, E. intergriceps (Terra et al. 1977; Chen et al. 1992; Oliveira-Neto et al. 2003; Kazzazi et al. 2005; Mehrabadi et al. 2011). The presence of different $\alpha$-amylase isoenzymes could be related to the importance of this enzyme in the insect food digestion.

Figure 4. Native-PAGE gel electrophoresis of $\alpha$-amylase in Rhynchophorus ferrugineus larvae.
It could be concluded that Red Palm Weevil has a mixture of two different isoenzymes which are active at acidic pH. The enzyme is active at a wide range of temperatures from 30 to over 50°C.

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