Article

Partial characterization of the digestive proteases and α-amylase of the larvae of the red palm weevil, *Rhynchophorus ferrugineus*

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Received 23 December 2019; *Accepted 10 January 2020*; *Published 1 March 2020*

Abstract
Activity of digestive enzymes (trypsin, chymotrypsin and α-amylase) in soluble protein extracted from the larval gut of *Rhynchophorus ferrugineus* was studied using specific substrates and enzyme inhibitors. Digestive enzyme activity showed a noticeable increase between day 5 and day 35. However, there was a noticeable decrease in the activity of trypsin protease on day 20. Maximum enzymes activity was observed at 40°C and pH of 8-9 in case of trypsin and chymotrypsin and pH 7-8 in case of α-amylase. Extract of digestive enzyme were incubated with different inhibitors specific for different classes of enzymes. SBTI and TPCK inhibited both trypsin and chymotrypsin like enzymes while could not inhibit α-amylase activity. EDTA and urea inhibitors could inhibit the activity of α-amylase but have no inhibition effect against trypsin and chymotrypsin enzymes. Kinetic analysis revealed higher *K*m value and higher *V*max value for the trypsin specific substrate (BA*p*NA) compared with the chymotrypsin specific substrate (BT*p*NA). On the other hands, the *V*max value recorded for BA*p*NA substrate was 11 times more than of that recorded for BT*p*NA substrate. The kinetic analysis of α-amylase like activity revealed that the enzyme has strong affinity to it substrate with moderate *V*max value. Results of the current study provide a fundamental knowledge that can be used to devise control strategies against *R. ferrugineus.*

Keywords *Rhynchophorus ferrugineus*; trypsin; chymotrypsin; α-amylase; enzyme inhibitors.

Arthropods
ISSN 2224-4255
URL: http://www.iaees.org/publications/journals/arthropods/online-version.asp
RSS: http://www.iaees.org/publications/journals/arthropods/rss.xml
E-mail: arthropods@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

Red Palm Weevil, *Rhynchophorus ferrugineus* (Oliver) (Coleoptera: Curculionidae), is a serious pest of a large number of plant species including, coconut, sago, oil and date palms. Red palm weevil was probably introduced in the Middle East through infested ornamental palm. It was first discovered attacking palm in the Arab peninsula, especially in the United Emirates in 1986, and slowly spread to the Gulf States and crossed the Red Sea into North Africa as the latest record in Egypt since 1992 (Abdel-Salam et al., 2014).

Some of the major aspects of pest control are actually achieving the selective suppression of many insect pests' digestive enzymes. The main objective of these attempts is to create detrimental effects on larvae growth

to prevent the absorption and assimilation of nutrients in order to delay their development and cause their death (Alarcon et al., 2002).

The biological activity of digestive enzyme inhibitors against insect pests was tested using three methods. The first and most direct method tests *in vitro* the ability of enzyme inhibitors to inhibit the function of enzymes from the gut of an organism (Abd El-latif, 2014a). The second method measures the target organism's response to protease inhibitors added to artificial diets (Kuroda et al., 1996; Abd El-latif, 2014b, 2015a, b, c). A third approach is to evaluate the response of insects to protease inhibitors in plant (Cloutier et al., 1999).

Characterization of the digestive enzymes of insects offers an open door for establishing appropriate and effective pest management strategies. There have been very less studies so far, on the characterization of digestive enzymes of *R. ferrugineus*, allowing the use of enzyme inhibitors as is the case with other insect species. In the current study the digestive enzymes (trypsin, chymotrypsin and α-amylase) were partially characterized during the development of *R. ferrugineus* larvae and the effect of different inhibitors on the activity of the digestive enzymes were also studied.

2 Materials and Methods

2.1 Insect culture

Adults and larvae of *R. ferrugineus* were collected from infested palm from Elwadi Elgedid Governorate, Egypt. Collected insects were reared on fresh sugarcane pieces in a growth chamber in at 25±0.5°C and 65±5% relative humidity. Larvae were individually reared in clear plastic containers. Five day old (L-1), 10 day old (L-2), 15 day old (L-3), 20 day old (L-4), 25 day old (L-5), 30 day old (L-6) and 35 day old (L-7) of the second generation larvae were used according to Alarco'n et al. (2002).

2.2 Preparation of larval gut homogenate

Gut enzyme extracts from L-2 to L7 of *R. ferrugineus* larvae was prepared according to the method of Johnston et al. (1993). The guts were dissected out and homogenized in ice-cold 0.2 M glycine-NaOH buffer, pH 8, containing 2 mM DTT and 10% PVP (10 guts/ml buffer). In case of the L-1 larvae the decapitated whole body larvae was used. The homogenates were kept for 2 h at 10°C and centrifuged at 10,000 rpm for 15 min at 4°C. The resultant supernatant was used as a source of digestive enzyme and stored at 20°C for further use.

2.3 Enzyme assays

2.3.1 Proteases assay

Trypsin and chymotrypsin like-activities were determined according to (Erlanger et al., 1961) using synthetic substrates BA*p*NA (N-benzoyl-dl-arginine-p-nitroanilide) and BT*p*NA (N-benzoyl-l-tyrosine-p-nitroanilide), respectively. Trypsin like-activity in the larval crude extract was measured by adding 1 ml of 1 mM BApNA in pre-warmed (37°C) 0.01 M Tris-HCl buffer (pH 8.0) containing 0.02 M $CaCl_2$ and incubated for 15 min at 37°C. Reactions were stopped by adding 200 µl of 30% glacial acetic acid. After centrifugation, the liberated p-nitroaniline in the clear solution was measured at 410 nm. Blanks, in which glacial acetic acid was added before the substrate, were prepared for each assay.

The chymotrypsin like-activity was also measured in a similar way except that the substrate used was BT*p*NA. One millimolar BTpNA was prepared in 0.01 M Tris-HCl (pH 8.0) containing 40% ethanol.

2.3.2 α-amylase assay

α-amylase like-activity was determined by the dinitrosalicylic acid (DNS) method using 1% soluble starch as substrate. Twenty microliters of the larval crude extract was incubated with 500µL sodium phosphate buffer (pH 7, 0.1 M) and 40 µL soluble starch for 30 min at 35°C. Hundred microliters of DNS was add to stop the reaction and then boiled for 10 min. After cooling the absorbance was read at 540 nm and one unit of α-

amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min. Maltose standard curve was constructed to enable calculation of the amount of maltose released during α-amylase assays.

2.3.3 Protein determination

Protein was determined following the method of Lowery et al. (1951) where bovine serum albumin was used as a standard.

2.4 Effect of temperature and pH on enzymes activity

The effect of temperature and pH on enzymes activity was determined using the crude extract of 30 day old larvae (L-6). The effect of temperature on enzymes activity was determined by pre-incubating of the reaction mixture at 20, 30, 35, 40, 50 and 60°C for 30 min followed by measurement of activity as described before.

The effect of pH on the enzymes activity of was assayed at different pHs which ranged from 2 to 12 using the following buffers at final concentrations of 0.1 M: glycine-HCl for pH 2 and 3; Na-acetate-acetic acid for 4 and 5; phosphate buffer for 6 and 7; Tris-HCl for 8; glycine-NaOH for 9 and 10, and CAPs buffer for pH 11and 12.

2.5 Effect of inhibitors on enzymes activity

The sensitivity of larval enzymes to specific and general inhibitors was studied by incubating the crude extract of 30 day old larvae (L-6) with different concentration of tested inhibitors for 60 minutes before adding the specific substrate and enzymes activity was measured as described before and results were expressed as IC_{50} relative to control without inhibitors. The inhibitors used were: PMSF (phenylmethylsulfonyl fluoride, a serine protease inhibitor), SBTI (soybean trypsin inhibitor, a serine protease inhibitor), TPCK (*N*-tosyl-l-phenylalanine chloromethyl ketone, a chymotrypsin inhibitor), EDTA (a metaloprotease inactivator), SDS (sodium dodecylsulfate), and urea.

2.6 Kinetic studies

Kinetics activity of the 30 day old larvae (L-6) enzymes was determined at different substrate concentrations using Lineweaver–Burk plots, in which the inverse of the initial velocity was plotted against the inverse of the substrate concentration, *K*m and *V*max were calculated.

3 Results

3.1 Enzymes activity

Table 1 shows changes in values of digestive enzymes (trypsin, chymotrypsin and α-amylase) activity found within the larvae gut from five days post-hatch until day 35. The trend of trypsin activity was an increase of activity with age up to 15 days (L-3) (0.92 U/mg protein), after which noticeable decrease of trypsin activity was found (L-4). The highest trypsin activity (0.99 U/mg protein) was observed in 30-day-old larvae (L-6).

Chymotrypsin activity was gradually increase from (0.20 U/mg protein) in L-1 larvae and reached it maximum (0.49 U/mg protein) in L-6 larvae. However sudden decrease in chymotrypsin activity (0.19 U/mg protein) was noticed in L-7 larvae. The trend of α-amylase activity was almost same as chymotrypsin activity, where L-6 larvae were observed to have the highest α-amylase activity (0.162 U/mg protein) compared to other larval ages.

3.2 Effect of temperature and pH on enzymes activity

Fig. 1 shows the optimum temperature for the activity of the digestive enzymes in *R. ferrugineus* larvae. Temperatures from 35-40°C seem to be suitable for the activity of all digestive enzymes tested however the highest activity was obtained at 40°C. The activity of trypsin and chymotrypsin enzymes decreased at 50°C while α-amylase activity did not significantly affect. At 60°C, α-amylase lost 68% of its activity.

Digestive enzymes were active over a broad range of pH from 2 to 12 (Fig. 2). However, the highest

activity was observed at pH range from 8-9 in case of trypsin and chymotrypsin enzymes and from 7-8 in case of α-amylase.

Table1 Activity of digestive enzymes during larval development of *R. ferrugineus.*

Larval stage	Enzyme activity (U/mg protein)		
	Trypsin	Chymotrypsin	Alpha amylase
L1	0.43	0.20	0.122
L2	0.73	0.31	0.102
L3	0.92	0.42	0.149
L4	0.69	0.41	0.152
L5	0.89	0.45	0.161
L6	0.99	0.49	0.162
L7	0.71	0.19	0.089

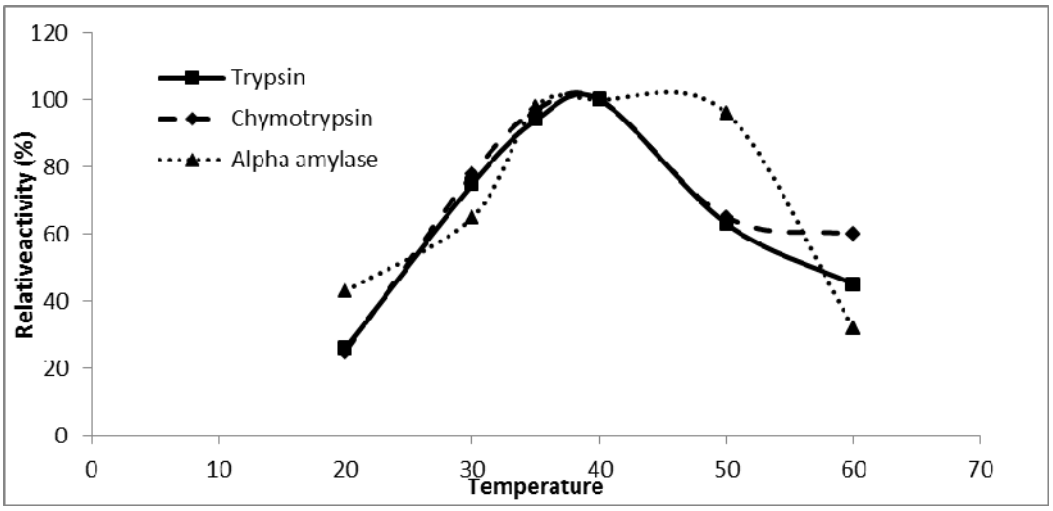

Fig. 1 Effect of temperature on the activity of digestive enzymes in in *R. ferrugineus*.

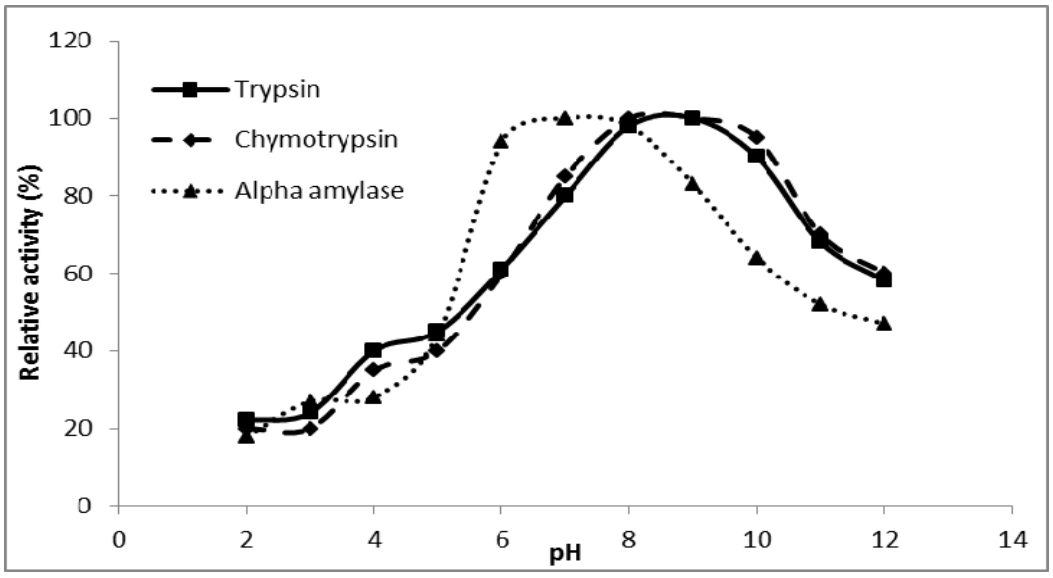

Fig. 2 Effect of pH on the activity of digestive enzymes in *R. ferrugineus*.

3.3 Effect of inhibitors on enzymes activity

Different concentrations of standard synthetic inhibitors PMSF, SBTI, TPCK, EDTA and SDS were used to determine the IC_{50} of digestive enzymes of *R. ferrugineus* gut extract. Results showed that in all cases linear inhibition of enzyme activity with increasing of inhibitor concentration until saturation was achieved. Calculated values of IC_{50} are presented in Table 2. Inhibition of trypsin activity using the trypsin specific inhibitors SBTI gave IC_{50} values of 4.12 µg/ml. PMSF, TPCK, EDTA and SDS have moderate inhibition activity against trypsin.

Highest inhibition potency against chymotrypsin enzyme was achieved when TPCK was used with IC_{50} values of 3.42 µg/ml. When urea inhibitor was used against trypsin and chymotrypsin enzymes, no inhibition activity was achieved.

Inhibition of α-amylase activity using the general inhibitors PSMF and EDTA gave IC_{50} values of 6.35 and 3.52 µg/ml, respectively. However, no α-amylase inhibition was achieved using SBTI and TPCK inhibitors. Urea had moderate inhibition potency against α-amylase with IC_{50} values of 13.71 µg/ml.

Table 2 Effect of different inhibitors on the activity of digestive enzymes in *R. ferrugineus.*

Inhibitor	IC_{50} (µg/ml)		
	Trypsin	Chymotrypsin	Alpha amylase
PMSF	12.43	10.23	6.35
SBTI	4.12	18.45	>100
TPCK	38.68	3.42	>100
EDTA	23.42	17.41	3. 52
Urea	>100	>100	13.71

3.4 Kinetic studies

Kinetic analysis of trypsin, chymotrypsin and α-amylase like activity gave line reciprocal Michaelis-Menton (Lineweaver-Burk) plots, enable the estimation of *K*m, *V*max values (Fig. 3). The kinetic analysis revealed higher *K*m value and higher *V*max value for the trypsin specific substrate (BA*p*NA) compared with the chymotrypsin specific substrate (BT*p*NA). The *K*m values were 1.24 and 1.03 mM for BA*p*NA and BT*p*NA substrates, respectively. On the other hands, the *V*max value recorded for BA*p*NA substrate was (11.97) which almost 11 time more than of that recorded for BT*p*NA substrate (1.11). These values indicate that trypsin specific substrate (BA*p*NA) has lower affinity to enzyme and is more rapidly hydrolyzed when saturated compared to the chymotrypsin specific substrate (BT*p*NA).

The kinetic analysis of α-amylase like activity revealed that the enzyme has strong affinity to it substrate with *K*m value of 0.24% while *V*max value was 0.85.

4 Discussion

Study on digestive enzymes of insects is one of the new and winning areas to reach a safe and effective way to decrease the damage of the pest on agricultural products (Mahdavi et al., 2013) and it offers an opportunity for developing appropriate and effective pest management strategies. If, enzymatic activities of such pest species are known then it will be helpful for formulating control strategies against this species. In the current study the activity of digestive enzymes in the larvae of *R. ferrugineus* was studied.

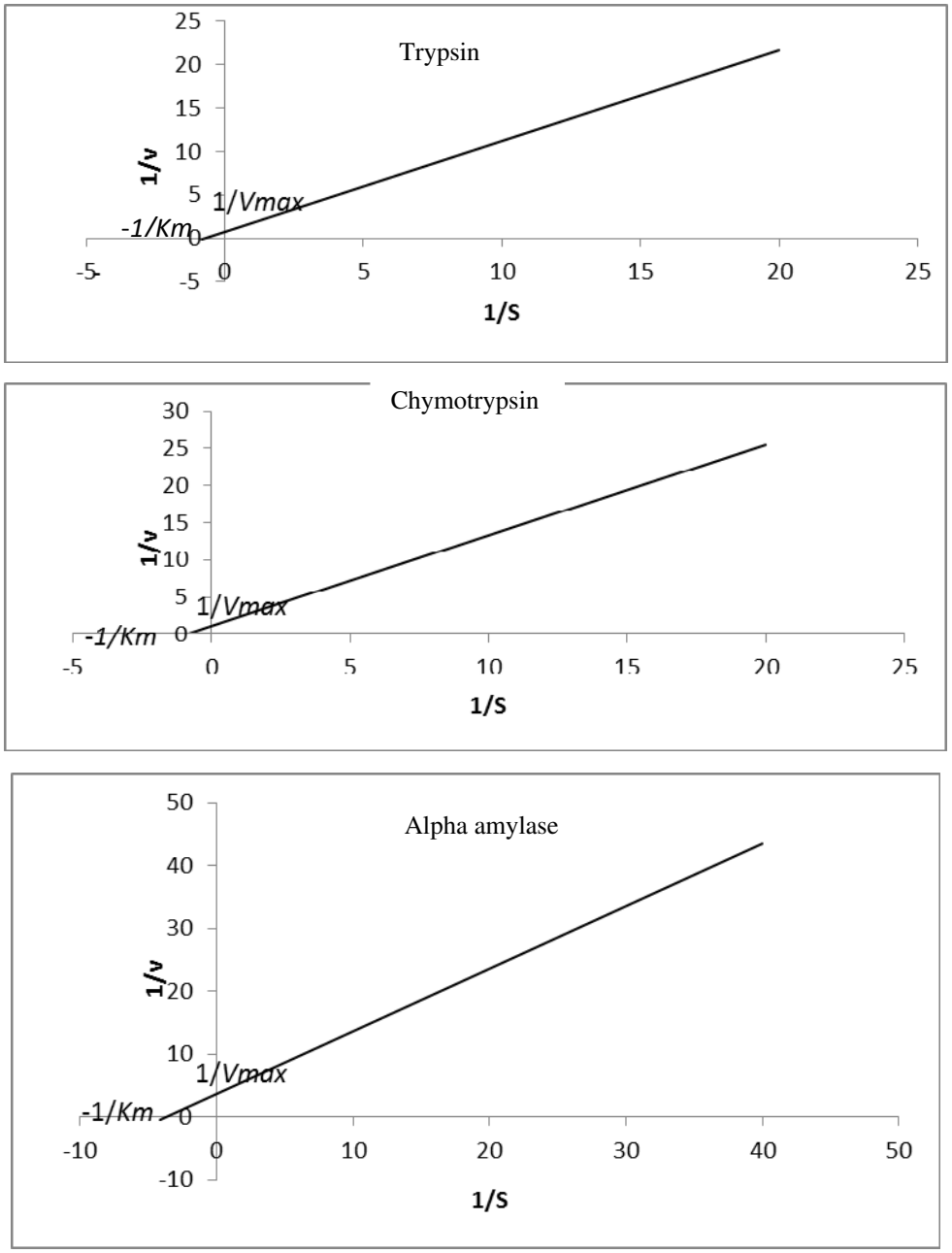

Fig. 3 Lineweaver–Burk plots for digestive enzyme in *R. ferrugineus*.

Digestive enzyme activity measured during the active larvae feeding phase expressed as U/mg protein, showed a noticeable increase between day 5 and day 35. Nonetheless, there was a noticeable decrease in the activity of trypsin protease on day 20.this decline may result from a greater degradation or a lower synthesis of digestive proteases resulting from quantitative reduction in the intake of feed when larvae are close to the next molt stage (Alarco'n et al., 2002).

Digestive enzymes of *R. ferrugineus* were active over a broad temperature range from 20 to 60°C. However the optimum temperature was recorded at 40°C.

Insects are adapted to a wide variety of diets and food digestion depends on the pH of the insect gut. Insects can precisely control the pH of the gut to provide optimum digestive system conditions (Alarco'n et al., 2002). Biggs and McGregor (1996) identified three-unit pH variability along the Coleoptera *Costelytra zealandica* digestive tract. Darvishzadeh et al. (2013) reported that gut pH of *R. ferrugineus* is

acidic (pH around 5). In this study the digestive enzymes of the 30-day larvae showed significant activity only at alkaline pH with pH (8-9) for trypsin and chymotrypsin and pH (7-8) for α-amylase. This high value is not the same as Darvishzadeh et al. (2013) reported the optimum pH. On the other hand an alkaline optimum pH was also reported by (Alarco'n et al., 2002).

Extract of digestive enzymes were incubated with different inhibitors specific for different classes of enzymes. The trypsin specific inhibitor (SBTI) and the chymotrypsin specific inhibitor (TPCK) inhibited both trypsin and chymotrypsin like enzymes while SBTI was stronger than TPCK against trypsin and TPCK was stronger against chymotrypsin. SBTI and TPCK could not inhibit α-amylase activity. EDTA and urea inhibitors could inhibit the activity of α-amylase but have no inhibition effect against trypsin and chymotrypsin enzymes. Similar effect was observed by (Alarco'n et al., 2002; Darvishzadeh et al., 2013). Based on the alkaline pH optimum of gut protease activity of R. ferrugineus and its inhibition by diagnostic protease inhibitors, Alarco'n et al. (2002) reported that the gut of the larvae contain mainly serine proteases. Terra and Ferreira (2005) reported that the *R. ferrugineus* α-amylase is sensitive to chelating agent (EDTA), which absorb metal ions, urea and SDS.

Measuring the catalytic activity of enzymes determines the physiological capacities of the different metabolic pathways. The kinetic parameters of digestive enzymes in *R. ferrugineus* have not studied before. In our study, kinetic analysis revealed higher *K*m value and higher *V*max value for the trypsin specific substrate (BA*p*NA) compared with the chymotrypsin specific substrate (BT*p*NA). On the other hands, the *V*max value recorded for BA*p*NA substrate was 11 times more than of that recorded for BT*p*NA substrate. These values indicate that trypsin specific substrate (BA*p*NA) has lower affinity to enzyme and is more rapidly hydrolyzed when saturated compared to the chymotrypsin specific substrate (BT*p*NA). The kinetic analysis of α-amylase like activity revealed that the enzyme has strong affinity to it substrate with moderate *V*max value.

The results of the current study provide a fundamental knowledge on the digestive capacity of *R. ferrugineus* for nutrient degradation that can be used to devise control strategies against the species being studied.

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