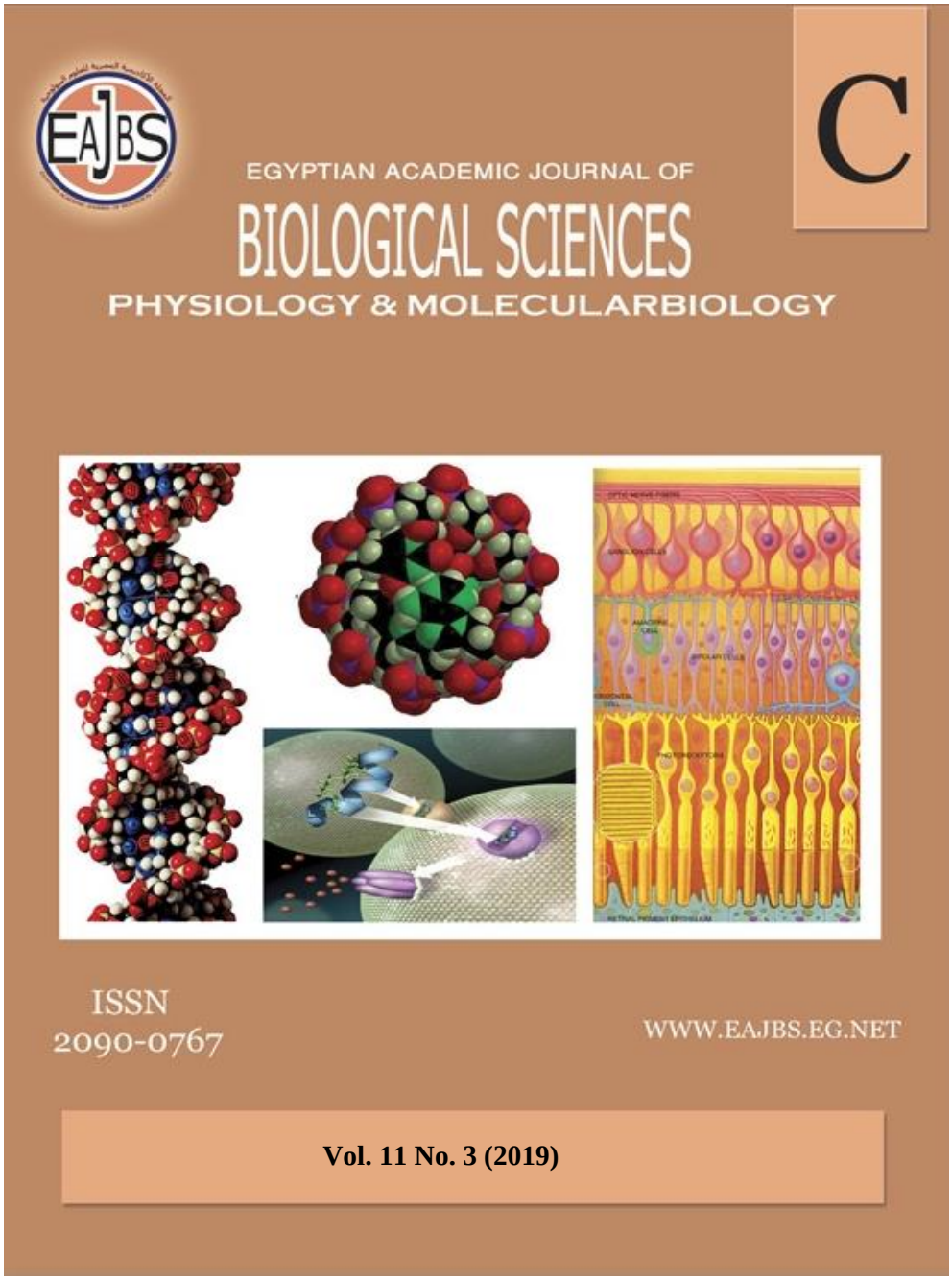

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Partial Kinetic Analysis of Haemolymph Esterases From The Red Palm Weevil; *Rhynchophorus ferrugineus* Oliv. (Coleoptera: Curculionidae)

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ABSTRACT

The paper represents a comprehensive study of the optimum subcellular fractions were prepared from the haemolymph of the seventh instar of the red palm weevil (RPW), *Rhynchophorus ferrugineus* Oliv. (Coleopter Curculionidae) to study some physicochemical characteristics of haemolymph esterases. The results showed that there were differences in characters between haemolymph α-esterases (hydrolyze alpha naphthyl acetate) and β-esterases (hydrolyze beta naphthyl acetate). The optimal pH and temperature were 8 and 50°C for α-esterases, respectively, and they were 7 and 35°C for β-esterases, respectively. Km (Michaelis constant) determined with alpha naphthyl acetate was $4X10^{-7}$ M, and it was $13.3X10^{-6}$ M for that determined with beta naphthyl acetate. V_{max} (maximum velocity) was 5.55 mg α-naphthol/min/mg protein and 1.66 mg β-naphthol/min/mg protein for α- and β-esterases, respectively. The effect of organic solvents on substrate hydrolysis was also discussed. The study provided biochemical optimized conditions for esterases activity, and with these ongoing studies, our further aim will be to develop new strategies for the red palm weevil control using disruptors of esterases as important detoxifying enzymes.

INTRODUCTION

The red palm weevil (RPW), *Rhynchophorus ferrugineus* Olivier, is the most dangerous Tissue-boring pest of the date palm, *Phoenix dactylifera* in many parts of the world and responsible for the death of a large number of palm trees causing dramatic yield losses for the last decades (Esteban-Duran *et al.*, 1998; Ferry and Gomez, 2002; Abozuhairah *et al.*, 1996 and Faleiro, 2006). It is the most important pest of the date palm in the Middle East (Abraham *et al.*, 1998). It was reported in Egypt infesting many governorates such as Ismailia and Sharkia, an area with an estimated one million palm trees (Salama and Abdel Aziz, 2001). Although they have been known as major pests for a long time, efficient and acceptable methods of controlling them are still lacking in many cases.

R. ferrugineus larvae are responsible for damaging the palm trees. They feed on tender tissues in palm trunks, which contain mostly lignocellulose and tree sap, alters the health of palm trees by leaving large tunnels, hollows and enormous brownish waste inside and eventually kills the trees (Nassar and Abdullah, 2001and Ferry and Gomez, 2002).

On the other hand, one of the most fundamental metabolic enzymes in different living organisms are esterases, they catalyze the hydrolysis of various endogenous as well as xenobiotic chain carboxyl esters among widely studied organisms including microorganisms, invertebrates as well as vertebrates. They considered as a large, diverse group of enzymes with wide, overlapping substrate specificities and patterns of inhibition (Raymond *et al.,* 1987; Karunaratne *et al.,* 1993; and Chen *et al.,* 2014).

Insect esterases are related to several metabolic processes, such as food digestion, a crucial role in toxic tolerance as well as degradation of insecticides thus devolving resistance, juvenile hormone hydrolysis, and other physiological activities such as the response to pheromone and plant volatiles (Campbell *et al.,* 2003). They also seem to be related to insect sexual activity (Richmond and Senior, 1991; Oakeshott *et al.,* 2010)

A wide range of different esterases exists in insects with variable substrate specificity and function. A large group of insect esterases that are usually active toward naphthyl ester substrates (Oakeshott *et al.,* 2010). Esterase isozymes in insects, particularly those in *Drosophila* sp. and mosquitoes, are classified according to their preferential hydrolysis of the isomeric artificial substrates, α-naphthyl acetate and β-naphthyl acetate (Oakeshott *et al.,* 1993). Although the classification has little value as a predictor of enzyme function, esterase activities measured using those substrates have often been proven to be associated with insecticide tolerance and resistance. (Zhu and Brindly (1990).

To our knowledge, no previous studies on RPW esterase characterization and kinetic activity were explored yet. Accordingly, the aim of this work was to study some *in vitro* physicochemical characteristics and kinetic properties of haemolymph α and β-esterases of *R. ferrugineus* larvae. Both types of esterases were compared from the following aspects 1) Their properties and kinetic characteristics, 2) the Effect of organic solvents on their catalysis 3) detection of the optimal conditions necessary for the quantitative determination of both α and β-esterases in the haemolymph of *R. ferrugineus* 7th instar larvae.

MATERIALS AND METHODS

Insects:

The red palm weevil, *R. ferrugineus* was collected as larvae from trunks of infested palm trees in Ismailia governorate, Egypt. When the insects were brought into the laboratory, they were bred at 25°C and fed on sugar cane stems till the 7th larval instar that chosen for esterases studies.

Chemicals:

Organic solvents were from Fluka chemical Gmb (Switzerland). Chemicals used for preparing different buffers were purchased from Aldrich chemical company (Milwaukee). The following chemicals are products from Sigma chemical company (St. Louis): coomasie brilliant blue G-250, bovine serum albumin, fast blue B salt, α- and β-naphthyl acetate, and α- and β-naphthols.

Collection of Haemolymph:

Haemolymph was collected from the 7th larval instar as described by Amin and Azazy (2008). The larvae were chilled at 4°C for about 5 min to become motionless. Then, quickly, Haemolymph was collected by

puncturing the first abdominal segment, and by gentle pressing on the abdomen, the blood flows into Eppendorf tube externally coated with ice to prevent melanization. Haemolymph esterases were fractionated to study their kinetic characters. Haemolymph containing blood cells, so it was fractionated by centrifugation at 105.000 X g for 90 min to obtain the microsomal pellet. Then, the pellet was decanted, and the supernatant or semi-purified fraction was stored at -20°C till the use.

Protein Assay:

Total protein in the haemolymph was determined by the method of Bradford (1976) using Coomasie brilliant blue G-250 as the dye binding to proteins, and bovine serum albumin as the standard.

Esterases Assay:

Gomori's colorimetric method, as modified by Van Asperen (1962), was used with α- and β-naphthyl acetate as substrates for α- and β-esterases (non-specific esterases), respectively. One ml of reaction mixture consisted of about 5 ug protein and α- or β-naphthyl acetate in the appropriate buffer. After incubation, the reaction was stopped by the addition of 200 ul fast blue β-SDS solution. Absorbance was read 15 min later at 600 and 555 nm for the produced α- and β-naphthol, respectively, against blank that lacked enzyme.

Esterases Partial Kinetics (V_{max} and K_m):

Alpha and β-esterases kinetic parameters were measured by mixing different concentrations of α- or β-naphthyl acetate as substrate (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, $10^{-1,}$ and 1 mM) with enzyme soluble fraction (contained 5 ug protein) and performing the steps as described above. The Michaelis constant (K_m) and maximal velocity of the reaction (V_{max}) were calculated by Sigma plot software. The data of K_m and V_{max} were fixed at the means ±SE of three replicates for each concentration.

Optimal pH of Esterases:

Measurement of esterases activity at different pHs was performed using different buffer values (4-10) to obtain the optimized pH. Other reaction conditions were at optimum found experimentally. The buffer system consisted of 0.1 M Citric acid (pH 4-5), 0.1 M Phosphate buffer (pH 6-8), 0.25 mM Tris-HCl (pH 9), and 0.05 M glycine-NaoH (pH 10). Three biological replications were used for each pH value.

The Optimal Temperature of Esterases Reaction:

Measurement of esterases catalyzed reaction in the range of 15-80°C was detected. Samples and reagents were pre-incubated at the tested temperature degree before the initiation of the reaction. The reaction of esterases activity proceeded as described above, and the highest enzyme activity was considered as optimum temperature.

Treatment by Organic Solvents:

Organic solvents may be used during enzyme preparation studies, so the effect of common solvents as acetone, ethanol, and methanol (Absolute, HPLC grade) on the two studied types of esterases was detected.

Serial concentrations of the solvents were prepared (0.1, 1, and 10 M) were prepared, then 100 µl of the tested solvent were added to about 5 µg protein of the enzyme solution and incubated for 5 min at the optimum temperature of the reaction. Then the esterases reaction was started by adding the substrate solution. The results were compared to the reaction mixture containing 100 ul of distilled H_2O instead of a solvent solution.

RESULTS

1-Optimum Conditions for Esterases Catalysis:

Optimum conditions for esterase reaction were detected by studying the influence of some factors

like pH, temperature, substrate concentration, and velocity of the reaction. The studied factors were variable, while other factors were at optimum.

Effect of pH:

Haemolymph esterases activity towards naphthyl acetate (substrate) was studied at 6 pHs values ranging from 4-10 (Fig.1). Alpha esterases did not show any activity at the acidic medium (pH 4-6). At a neutral medium (pH 7), the activity appeared suddenly. It was 7000 ug α-naphthol/min/mg protein. Then the activity reached its peak at pH 8 (10500 ug α-naphthol/min/mg protein). At more alkaline media, α-esterases were active but showed a moderate decrease as compared to that of the optimum. It was 7300 and 6500 ug α-naphthol/min/mg protein at pH 9 and 10, respectively.

β-esterases showed some differences from α-esterases. They showed little activity at acidic medium (pH 6), and the optimum activity was at pH 7 and began to decrease at alkaline pH ^. It was 2440, 2320, 2100 and 2000 β-naphthol/min/mg protein at pH 7, 8, 9 and 10, respectively. The results showed that the major activity of esterases was at neutral and alkaline media.

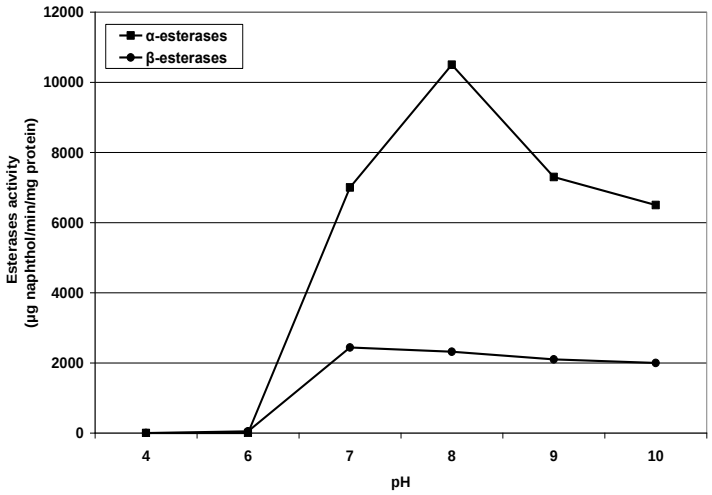

Fig. 1: Effect of reaction pHs on haemolymph esterases activity in the 7th larval instar of the red palm weevil, *R. ferrugineus.* Each point represents the mean of 3 determinations (means±SE). Reaction time was 10 min at 50 and 35°C for α-esterases and β-esterases, respectively.

Effect of Temperature:

The data in Figure (2) shows the effect of temperature (15-80°C) on enzyme catalysis. The activity of α-esterases sharply increased when the temperature rose from 15 to 50°C, where the optimal temperature was reached. It was 2660, 3948, 4172 and 8596 µg α-naphthol/min/mg protein at 15, 25, 35 and 50°C, respectively. Alpha esterases lost their most activity at 80°C, where the activity decreased by 72.2% less than the optimal activity (at 50°C).

The optimum temperature for β-esterases was less than that of α-esterases. The optimal temperature of β-esterases was 35°C. Also, β-esterases resisted higher temperature degrees i.e. they were more thermostable than α-esterases. The activity in the range from 35-60°C showed non-significant differences ($P<0.01$). It was 3600, 3300, and 3000 β- naphthol/min/mg protein at 35, 50 and 60°C, respectively. It is likely to note that at the highest temperature (80°C), the enzyme protein was still active as compared to enzyme catalysis at the optimum temperature.

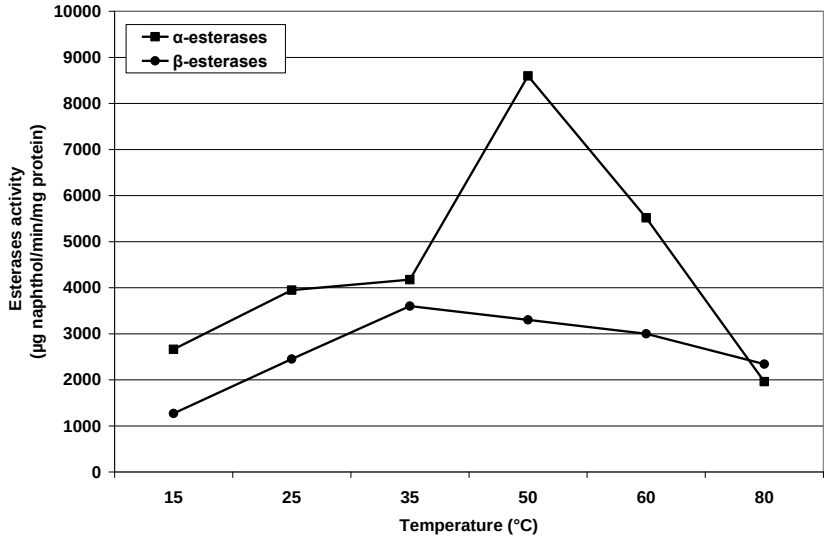

Fig. 2 : Effect of reaction temperature on haemolymph esterases activity in the 7th larval instar of the red palm weevil, *R. ferrugineus.* Each point represents the mean of 3 determinations (means±SE). Reaction time was 10 min at pH 8 and 7 for α-esterases and β-esterases, respectively.

Esterases Reaction Time:

Thirty minutes allowed for esterases catalysis to determine the suitable time allowed for reaction (Fig.3). The reaction rate of haemolymph α-esterases of *R. ferrugineus* larvae was directly proportional to the first five minutes of the reaction. Whenever the reaction time was doubled, the catalysis increased in the same manner. The enzyme activity was 1596, 2303, 3688 and 7133 α-naphthol/min/mg protein after 1, 2, 3 and 5 min, respectively. After 5 min from reaction proceeding, the catalysis was very low during the rest of the experiment. The reaction rate of β-esterases was found to be more or less constant and directly proportional to time of the experiment (30 min).

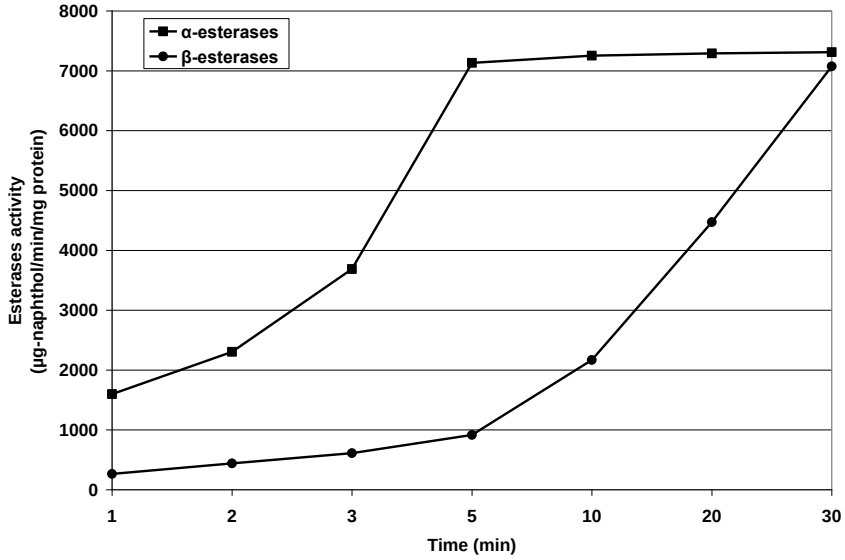

Fig. 3 : Effect of experiment time on the reaction rate of haemolymph esterases of the red palm weevil, *R. ferrugineus.* Data are presented as the mean±SE (n=3). Optimum pH and temperature were followed.

Effect Substrate Concentration on Esterases Catalysis:

Effect of substrate concentration on haemolymph esterases activity of *R. ferrugineus* larvae was studied using 7 concentrations ranged between 10^{-2} to 10^{-8} M naphthyl acetate (substrate) in one ml of the reaction mixture (Fig. 4). Alpha esterases activity was peaked at 10^{-4} M α-naphthyl acetate, while β-esterases (Fig. 5) activity peak was at higher substrate concentration (10^{-3} M β-naphthyl acetate). Substrate concentration higher than 10^{-4} M caused substrate inhibition to α-esterases. Enzyme activity was 11200, 7252, and 6000 µg α-naphthol/min/mg protein at 10^{-4}, 10^{-3} and 10^{-2} M α-naphthyl acetate, respectively. Substrate concentrations below 10^{-4} M α-naphthyl acetate and 10^{-3} M for β-esterases were low, and the activity was decreased to the extent that β-esterases had minor activity equaled to 30 µg α-naphthol/min/mg protein at 10^{-8} M of β-naphthyl acetate.

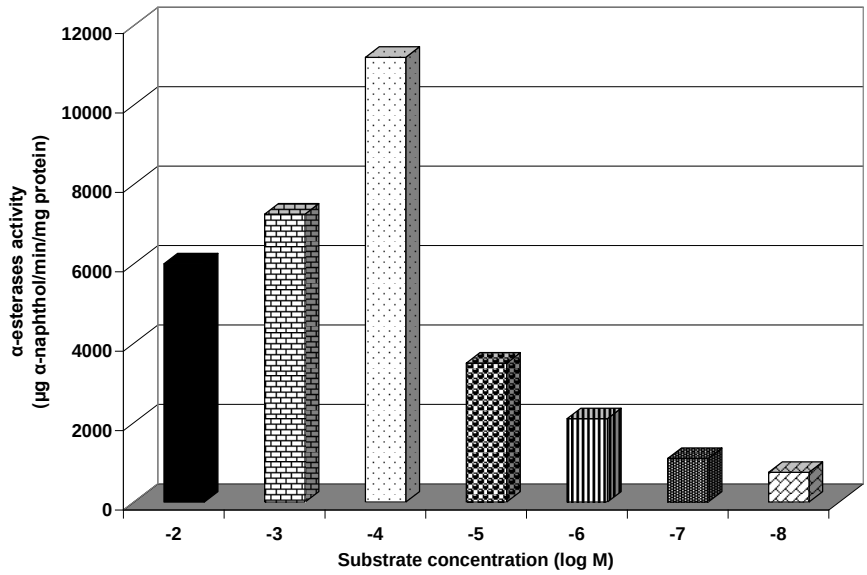

Fig. 4 : Effect of substrate concentration on haemolymph α-esterases catalysis of the red palm weevil, *R. ferrugineus.* Data are presented as the mean±SE (n=3). Optimum conditions of the reaction were followed.

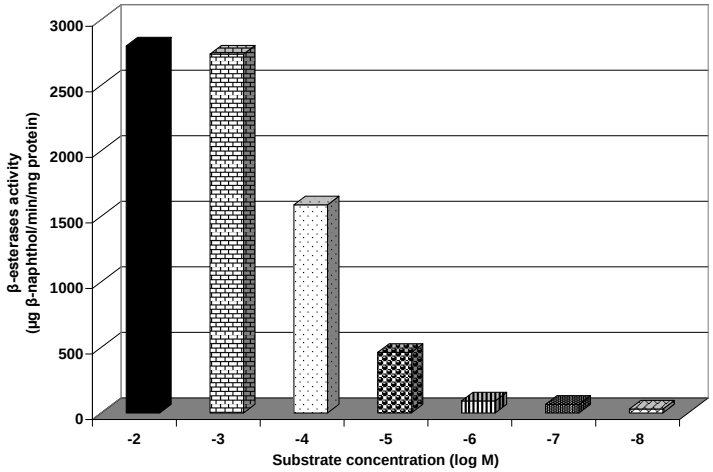

Fig. 5 : Effect of substrate concentration on haemolymph β-esterases catalysis of the red palm weevil, *R. ferrugineus.* Data are presented as the mean±SE (n=3). Optimum conditions of the reaction were followed.

2-Michaelis-Menten Kinetics of Esterases:

The reaction kinetics of haemolymph esterases from the 7th instar larvae of the red palm weevil were detected using Linweaver-Burk plot. When the linear reciprocal plot is extrapolated for α-esterases, it intersects the negative portion of the abscissa at -2.5 um (Fig. 6). Thus, K_m of α-esterases catalyzed reaction is $4X10^{-7}$ M, and V_{max} is 5.55 mg α-naphthol/min/mg protein. The graph (Fig. 7) constructed for β-esterases, shows that K_m of β-esterases is $13.3X10^{-6}$ M, indicating that substrate concentration at half maximum velocity is higher than α-esterases. On the other hand, V_{max} of β-esterases is lower than the other type of esterases, where it calculated 1.66 mg β-naphthol/min/mg protein.

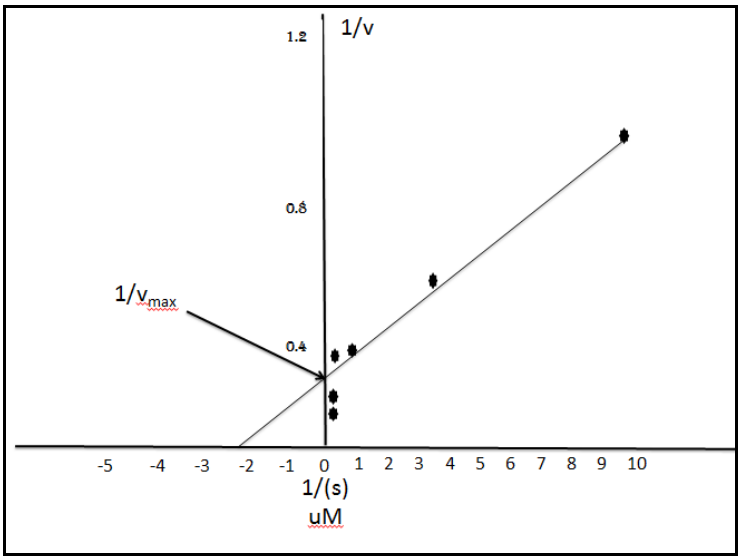

Fig. 6 : Double reciprocal (Lineweaver-Burk) plot of 1/V versus 1/(S) for the reaction catalyzed by α-esterases from *R. ferrugineus* larvae at pH 8, 50°C.

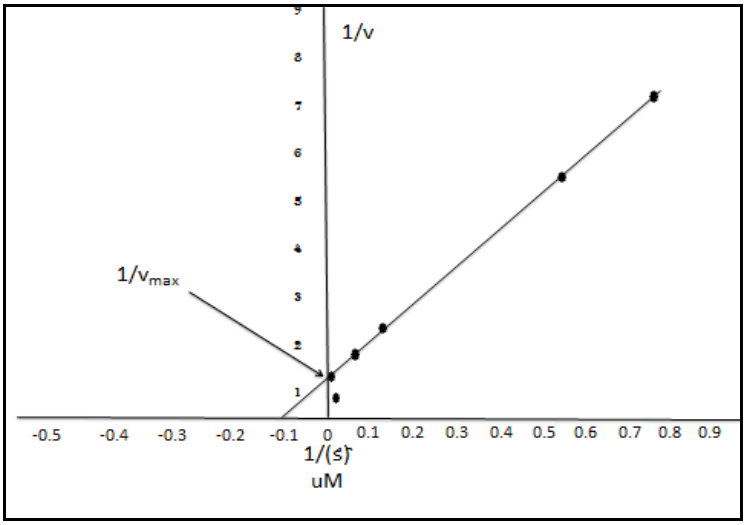

Fig. 7 : Double reciprocal (Lineweaver-Burk) plot of 1/V versus 1/(S) for the reaction catalyzed by β-esterases from *R. ferrugineus* larvae at pH 7, 35°C.

3- Effect of Organic Solvents:

The effect of pre-incubation of esterases with different concentrations of organic solvents on esterases catalysis is illustrated in Table (1). The results revealed that 10 M and I M of acetone and ethanol significantly activated α-esterases, while β-esterases activated only by 10 M of both solvents. 0.1 M concentration had no effect. On the contrary, incubation of the enzyme with 100 µl of 10 M methanol, significantly inhibited both α and β-esterases, while I M methanol slightly activated α-esterases catalysis.

Table1: Effect of incubation with different organic solvents on haemolymph esterases activity of the red palm weevil, *R. ferrugineus 7 th* instar larvae**.**

Solvents		α-esterases (α-naphthol/min/mg protein)	β-esterases (β-naphthol/min/mg protein)
Acetone	10 M	9200 ± 10^{b}	3500 ± 6^{b}
	1 M	8150 ± 6^{c}	2500 ± 3.3^{c}
	0.1 M	7100 ± 9^{e}	2390 ± 12^{c}
Ethanol	10 M	10200 ± 14^{a}	4000 ± 5.8^{a}
	1 M	7920 ± 5.5^{d}	2490 ± 3.7^{c}
	0.1 M	6900 ± 10^{e}	2230 ± 6.3^{c}
Methanol	10 M	4510 ± 10^{f}	1900 ± 6^{d}
	1 M	7600 ± 7^{d}	2300 ± 9^{c}
	0.1 M	7100 ± 8^{e}	2190 ± 5^{c}
Control		7100 ± 12^{e}	2420 ± 10^{c}

- Data are presented as the mean±SE
- Means, within column, bearing different subscripts are significantly different ($P<0.01$, ANOVA)

DISCUSSION

The present results showed that the major activity of esterases in *R. ferrugineus* haemolymph was at neutral and alkaline media coincide with optimal pH profile of esterases of most insect species; The optimal pH ranged between 7 and 8 in gypsy moth, *Lymantria dispar* (Kapin and Ahmad, 1980); *Culex tarsals* (Matsumura and Brown, 1963); *D. mulleri*,(Srinivas *et al.*, 2006); *D. repleta* (Lopes *et al.*, 2014); *Musca domestica* (Van Asperen, 1962); while the pH ranged between 6 to 7.5 in diamondback moth (He, 2003) and from 6.5 to 8.5 for *Periplaneta Americana* (Cook and Forgash, 1965).

The present work showed that the optimum temperature for α-esterases was at 50°C, while that of β-esterases was 35°C. It was found also that β-esterases resisted higher temperature degrees i.e. they were more thermostable than α-esterases.

Kapin and Ahmad (1980) reported that naphthyl esterase activity of the gypsy moth larvae gut was apparent up to 50°C, therefore, dropping sharply, presumably due to enzyme denaturation while the optimum temperature was (37°C) in both *Myzus persicae* (Sudderuddin, 1973) and in *P. americana* (Hipps and Nelson, 1974). In *D. fructuum* larva, the optimum temperature was 40°C while in *D. mulleri*, esterases had an

optimal activity in temperatures ranging from 40° to 45°C (Lopes *et al.,* 2014). He *et al.,* (2003) reported that the optimum temperature for measurement of diamondback moth esterase activity was in the range of 33–42°C and no enzyme activity was observed at 5°C or at 60°C at which the enzyme was apparently thermally denatured. In *Helicoverpa armigera*, the esterases isozymes were unstable at temperature more than 50 °C.(Srinivas *et al.,* 2006).

Accordingly, haemolymph esterases of *R. ferrugineus* in the present work showed a relatively higher thermostability rather than many insect species. This may be due to the presence of high temperatures throughout the year where palm trees are cultivated and may their esterases have a molecular structure more suited to this type of environment. A similar conclusion was achieved in the case of the stingless bees, *Tetragonisca weyrauchi* (Ronqui *et al.*, 1983).

In the present work, K_m of α-esterases and β-esterases in RPW were $4X10^{-7}$ and $13.3X10^{-6}$ M, respectively. In the gypsy moth, *L. dispar* (L.), larval tissues using 1-naphthyl acetate as substrate, the Km was determined to be 4.25×10^{-5} M (Kapin and Ahmad, 1980) while Km value of esterases in *Hyalomma dromedarii* was 1.43 mM (Fahmy *et al.,* 2004). In *Pieris brassicae*, Km values were reported as 2.72 mM (Zibaee, 2012).

The present data also showed that Vmax was 5.55 and 1.66 mg α-naphthol/min/mg protein for α and β-esterases, respectively. In *L. dispar*, V*max* reached 942 nmoles mg^{-1} min^{-1} at 30°C. The hydrolysis rate was linear for the first 25–30 min (Kapin and Ahmad, 1980) while in P. *brassicae*, Vmax was reported as 30.3 U/mg protein (Zibaee, 2012). It seems that the present results may indicate that both α and β-esterases relatively hydrolyze their substrates efficiently, even at very low concentrations, and have a high affinity to their substrates.

The present data showed that the effect of pre-incubation of esterases with different concentrations of organic solvents on esterases catalysis revealed that both acetone and ethanol significantly activated α-esterases, while β-esterases activated only by higher concentrations of both solvents. Also, incubation of the enzyme with 100 µl of 10 M methanol significantly inhibited both α and β-esterases, while I M methanol slightly activated α-esterases catalysis.

It seems that the catalytic efficiency of many enzymes is affected by organic solvents in different ways. In *Manduca sexta,* the juvenile hormone esterase JHE showed activation in different organic solvents (Browder *et al.*, 2001). The JHEs from coleopterans such as *Leptinotarsa decemlineata* and *Ips typographus*, is completely or partially inhibited by low (e.g., 0.1%) concentrations of Triton X- 100 (Kramer and De Kort, 1976 and Stauffer *et al.*, 1997).

Kamita *et al.,* 2003 reported that in the presence of acetone, enzymatic activity towards the ester bond of JH would be detected as JH hydrolysis. However, in the presence of higher alcohols, the enzyme activity may simply be shifted towards transesterification (McDonald and Balls, 1956). Grieneisen *et al.* (1997) reported that the JHE enzymes are actually capable of transesterification in the presence of ethanol or 1-propanol.

In general, non-specific esterases represent a large, diverse and complex group of major hydrolytic enzymes and possess the property of overlapping substrate specificity, hydrolysing both endogenous and exogenous esters of widely differing structures leading to problems of identification and classification (Walker and Mackness, 1983).

This study provides important baseline data that will assist in the understanding of esterases characterization of RPW is an essential

step for the elucidation of their biochemical mechanisms in developing their resistance and accordingly, offers an opportunity for developing appropriate and effective pest management strategies.

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