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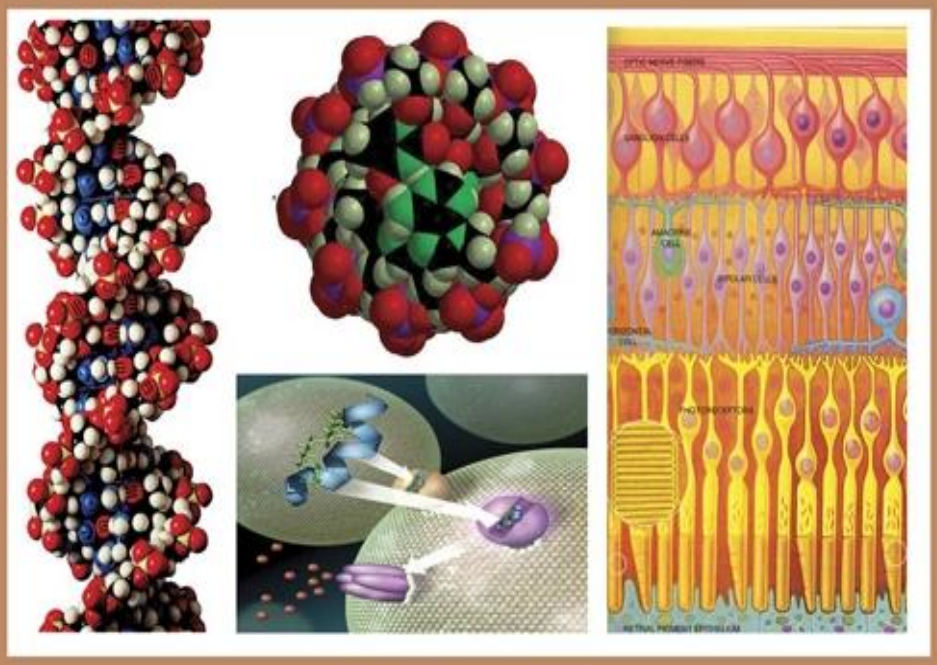

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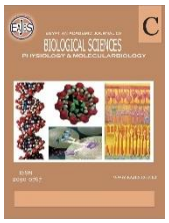

Characterization of Phenoloxidase in The Red Palm Weevil; *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) (Olivier) Using Catechol Substrate

Tarek R. Amin[1] and Nedal M. Fahmy[1&2]

1- Department of Pest Physiology, Plant Protection Research Institute (PPRI), Agricultural Research Center (ARC), Dokki, Giza, Egypt.

2-Department of Biology, University collage of Tayma, University of Tabuk (UT), Kingdom of Saudi Arabia (KSA).

*E. Mail: nedalashraf33@hotmail.com

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ABSTRACT

Phenoloxidases (EC.1.14.18.1) are implicated in the immunity of insects toward microorganisms, so oxidative enzymes such as phenoloxidases (PO) from the 7th instar larvae of the red palm weevil; *Rhynchophorus ferrugineus* (Oliv.) was partially isolated to characterize its activity using catechol as a substrate. The mitochondrial fraction of larval homogenate was used as enzyme source for partial kinetic studies.Enzyme catalysis was directly proportional to enzyme crude protein conc. up to 200 ug contained in 1 ml of the reaction mixture. Michaelis-Menten kinetics of PO activity was evaluated by constructing Lineweaver Burk double reciprocal plot. Michaelis constant (Km) was, 3.45 $X10^{-4}$ M and maximum velocity (V_{max}) of PO reaction was 344.8 $O.D.min^{-1}$ mg $protein^{-1}$. Effect of pH, temperature and substrate concentration on enzyme reaction was tested. Generally, each variable was chosen while other conditions were at optimum found primarily. Reaction rate was optimal at 10^{-1} N catechol, neutral medium (pH 7) and 40 C. PO kept its most activity when incubated at 60 C for 15 min before reaction initiation. Phenoloxidases efficiently catalyze catechol. Results in the present study may probably offer a fundamental anchor for future alternative strategies in controlling *R. ferrugineus* via obstructing its innate defence mechanisms.

INTRODUCTION

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier, 1790), is one of the most voracious and destructive pest invading palm species worldwide, specially the Middle Eastern countries (Abozuhairah *et al.*, 1996; Esteban-Duran *et al.*, 1998; Ferry and Gomez, 2002 and Faleiro, 2006). This pest invaded the Arabian Gulf countries in the mid-1980s (Abraham *et al.*, 1998 and Murphy and Briscoe, 1999). It has been recorded in Saudi Arabia and United Arab Emirates in 1985 (Ferry and Gomez, 2002) and later in Egypt in 1990 (Cox, 1993). The weevil is a threatening problem because date palm is one of the main sources of farmers' income in Arabian Gulf and North Africa (El-Sabea *et al.*, 2009). *R. ferrugineus* larvae feed on the inner tender tissues in palm trunks, searching for lignocellulose and tree sap.

The hidden mode of life of these grubs results in difficulty in applying different appropriate control strategies. The control of palm weevil is currently dependent on several methods such as pheromone traps (Navarro-Liopis *et al.*, 2018) as well as relying on limited biological control agents (EL Roby 2018; Ishak *et al.*, 2020 and Liu *el al.*, 2021).

The most common preventative measure is usually carried out via pesticide application as a quick solution in spite of hazards, natural enemies and environment (Faleiro, 2006) as well as their enhancement of pest resistance (Liu *et al.*, 2021).

Phenoloxidase (PO) which is a copper-containing enzyme (Kim and Uyama, 2005) is considered as one of the most important enzymes that have a major role in the insect innate immune system. It plays several key roles during metamorphosis having different physiologically important processes, including cuticle sclerotization, encapsulation and melanization of pathogens as well as wound healing (Ratcliffe *et al.*, 1984; Aso *et al.*, 1985; Ashida and Brey, 1995; Cerenius *et al.*, 2008; Kramer *et al.*, 2001 and Stączek *et al.*, 2020). It is responsible for the production of o-quinones which are the initial step in the biochemical cascade of cuticle sclerotization, quinone tanning and melanin biosynthesis and involved in several major roles in insect immunity and development (Chen *et al.*, 2003 and Klowden, 2007).

Phenoloxidase has been purified and characterized using different terms such as optimum PH and temperature, enzyme thermostability and inhibition in a number of different insects in several insect species including Blattodea, Orthoptera, Hemiptera, Lepidoptera, Diptera and many others (Ashida 1971; Yamaura *et al.*, 1980; Aso *et al.*, 1985; Ourth, 1988; Lockey and Orth, 1992; Fujimoto *et al.*, 1993; Burks and Fuchs 1995; Hall *et al.*, 1995; Kop á cek *et al.*, 1995; Lee and Anstee, 1995; Anderson *et al.*, 1998; Chase *et al.*, 2000; Wang *et al.*, 2004; Zufelato *et al.*, 2004; Hartzer *et al.*, 2005; Xue *et al.*, 2006; Feng *et al.*, 2008; Wang *et al.*, 2010; Zibaee *et al.*, 2011; Demir *et al.*, 2012 and Delkash-Roudsari *et al.*, 2015). However, to our knowledge, no previous studies had been focusing on characterizing PO in *R. ferrugineus*, the most threatening palm pest ever. This may help in designing new methods of control via inhibitors or altering the insect defense mechanisms and/or forcing the insect into abnormal body softening which could be used potentially in pest control as an alternative novel choice rather than environmentally threatening pesticides (Xue *et al.*, 2006 and Fahmy and Amin, 2019).

Accordingly, the current study aims to partially characterize PO for the first time in *R. ferrugineus* and elucidate the optimum conditions of its activity and determine some of its kinetic properties using catechol as a substrate.

MATERIALS AND METHODS

Insects:

The red palm weevil, *R. ferrugineus* larvae was collected from trunks of infested palm trees in El-Kasaseen farms, Ismailia governorate, Egypt. The collected insects were brought into the laboratory then, they were bred at 25°C and allowed to feed on sugar cane stems till the 7th larval instar for PO studies.

Chemicals:

The substrate, catechol and bovine serum albumin were purchased from Sigma chemical company (ST. Louis). Other needed chemicals for buffers were purchased from local companies.

Apparatus:

1.Homogenizer: Larvae were homogenized in chilled glass Teflon tissue homogenizers (ST-2 Mechanic-preczyina, Poland).

2. Spectrophotometer: Double beam ulteraviolet/visible spectrophotometer (spectronic 1201, Milton Royco., USA) was used to measure absorbance of metallic compounds or coloured substances.

Insect Sample Preparation:

Larvae were homogenized in distilled water (50 mg/ml). The insect homogenates were centrifuged for 15 minutes at 5^{o}C in a refrigerated centrifuge at 8000 r.p.m. The supernatant kept frozen at -20°C in a deep freezer till use.

Kinetic Properties of Phenoloxidase System:

Phenoloxidase activity was determined according to Ishaaya (1971) with modifications made by Amin *et al.,* (2013). The reaction mixture consisted of 0.5 mL phosphate buffer (0.1 M), 200 µl of Catechol solutions as substrate concentration and reaction temperature was determined to detect the optimum conditions of the reactions.

Prior to the reaction's initiation, the substrate and all needed ingredients were separately incubated at optimum temperature of the reaction. Phenoloxidase activity absorbance was recorded at a one-minute interval for 10 minutes against sample blank as the zero adjustment at 405 nm. Enzyme activity was expressed as Δ O.D. units $\times$ 10^{-3} min^{-1} mg $protein^{-1}$.

Total protein was determined for each sample according to Bradford (1976) using bovine serum albumin as slander. Mitochondrial fraction of larval homogenate was the enzyme source for partial kinetic studies such as the initial velocity.

Michaelis-Menten kinetics as well as the effect of pH, temperature and substrate concentration of PO activity and the highest enzyme activity was considered as optimum parameter value.

Phenoloxidase activity was studied by measuring seven concentrations of Catechol as a substrate between 10^{-1} and 10^{-6} M.

Optimal pH of PO activity at different pHs was performed using different buffer values (5-9) to obtain the optimized pH. Other reaction conditions were at optimum found experimentally. Three biological replications were used for each pH value.

The optimal temperature of PO activity was measured in the range of 5-60°C. Samples and reagents were pre-incubated at the tested temperature degree before the initiation of the reaction. Thermostability of PO was tested at 60 and 70^{o} C

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.

Data Analysis:

Obtained results were pooled as triplicate. Using coStat statistical software (Cohort software, Brekeley), Data were analysed using a completely randomized one-way ANOVA test. Means were separated using Duncans range tests ($P<0.01$).

RESULTS AND DISCUSSION

Enzymatic reactions like PO cascade and some of the non-enzymatic reactions lead to melanin formation from quinone at the final step of nodulation and encapsulation. Additionally, PO plays a key role in melanin production during cuticle sclerotization at external wound sites and during defense responses, (Mason, 1955; Ratcliffe *et al.*, 1984; Lokstan and Li, 1988; Zue *et al.*, 2006; Cerenius *et al.*, 2008 and Gholami *et al.*, 2013).

The present work studies some kinetic properties of phenoloxidase in the 7^{th} instar larvae of *R. ferrugineus* such as the effect of substrate concentration, pH, temperature, enzyme thermostability, reaction time, Km and V_{max}. Therefore, we can detect optimum condition of each factor separately for this enzyme while all other factors at optimum.

Reaction Time:

The reaction of PO was allowed to proceed for 60 minutes to determine the suitable time needed for this reaction. Results indicated that the linear part of PO reaction rate was during the first 3 min from reaction starting as shown in Figure 1. In the first 20 min, PO was able to retain about 85% of its activity, but enzyme activity fell to less than 40% after an hour of incubation at the optimal temperature.

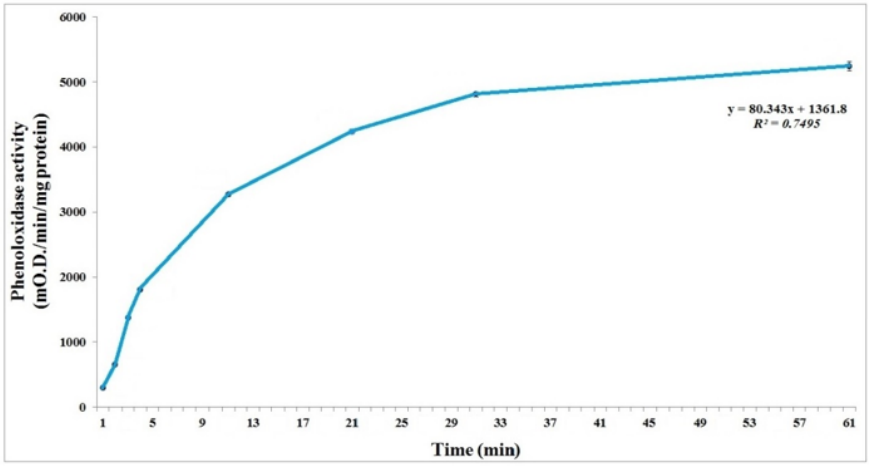

Fig.1: Effect of experiment time on the reaction rate PO of *R. ferrugineus*. Data are presented as the mean±SE (n=3). Optimum pH and temperature were followed

Effect Substrate Concentration:

Phenoloxidase activity was studied by measuring seven concentrations of Catechol as a substrate as shown in fig.2 between 10^{-1} and 10^{-6} M (Fig.2). The present data showed that 0.1 N catechol in the reaction mixture was the optimum concentration for enzyme activity. Below 0.01 N catechol, reaction rate declined sharply but at 10^{-3} to 10^{-6} showed a steady but relatively low rate.

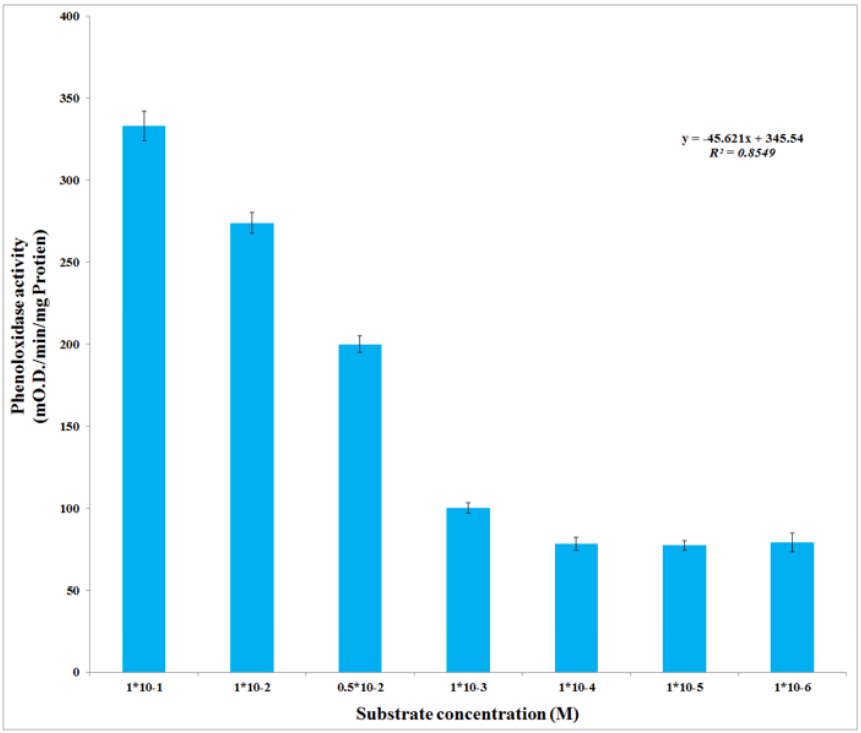

Fig. 2: Effect of substrate concentration on PO catalysis of *R. ferrugineus*. Data are presented as the mean±SE (n=3). Optimum conditions of the reaction were followed.

Effect Enzyme Concentration:

Phenoloxidase activity showed a linear increase up to 200 ug enzyme protein as shown in Figure 3.

Effect of pH:

Effect of pH upon the oxidation of catechol at ranging between 5 to 9 was indicated in Figure 4 showed a bell-shaped

curve with optimum activity at neutral point i.e., the highest activity of PO lies in neutral and slight acidic media. It starts to decline after pH 8 reaching its lowest value at pH 9.

Effect of Temperature And Thermostability:

Phenoloxidase activity toward catechol was studied also at seven values of temperature between 5 and 60^0 C. The enzyme had a relatively broad range of temperature degrees (25-60), where the change of activity was relatively low. The optimum temperature was 40 ^{0}C (Fig.5).

Thermostability of PO in *R. ferrugineus* in the present work was tested at 60 and 70^o C and results as shown in Figure 6 indicated that PO was thermostable. Incubation of PO at high temperatures for 15 min before initiation of reaction did not highly affect enzyme activity.

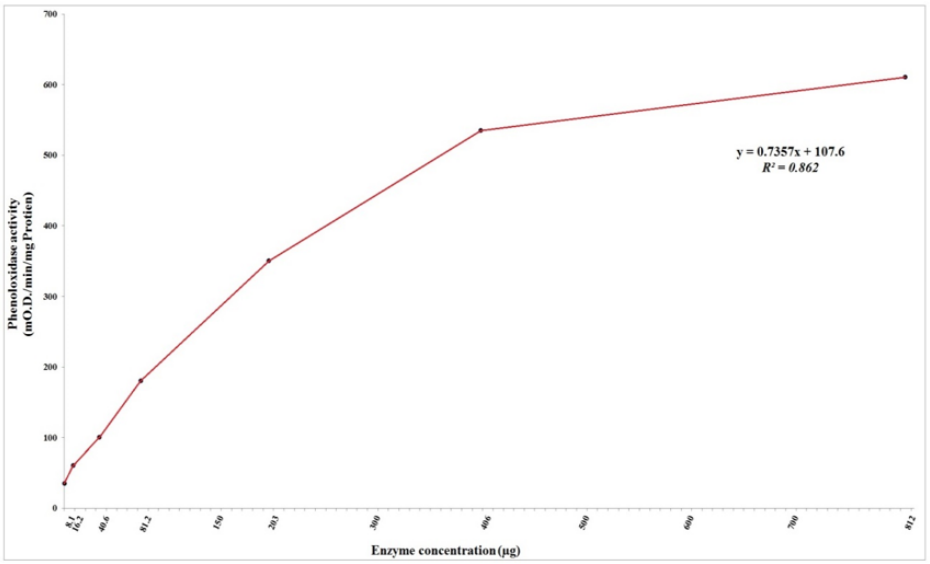

Fig. 3: Effect of Enzyme concentration on PO catalysis of *R. ferrugineus*. Data are presented as the mean±SE (n=3). Optimum conditions of the reaction were followed.

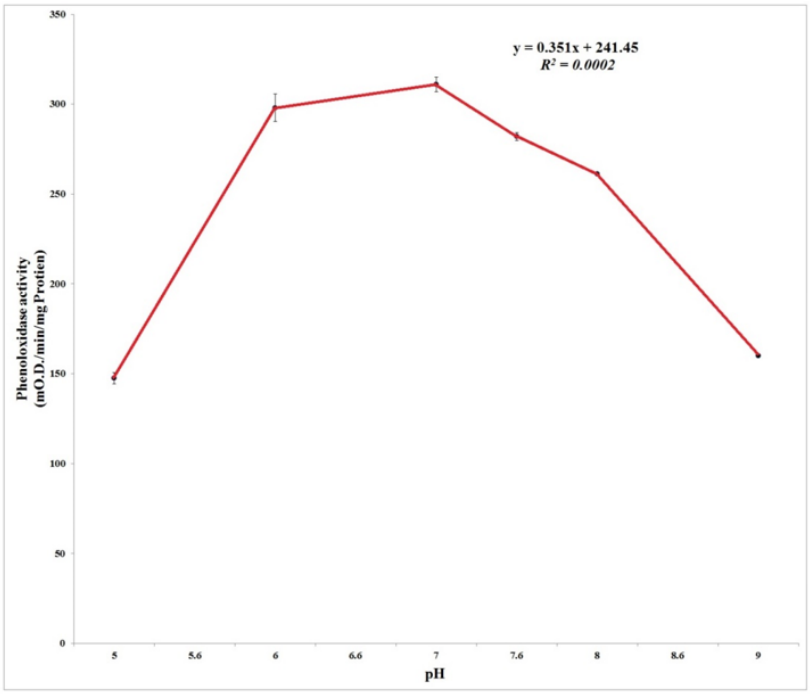

Fig. 4: Effect of reaction pHs on PO activity in the 7th larval instar of *R. ferrugineus*. Each point represents the mean of 3 determinations (means±SE).

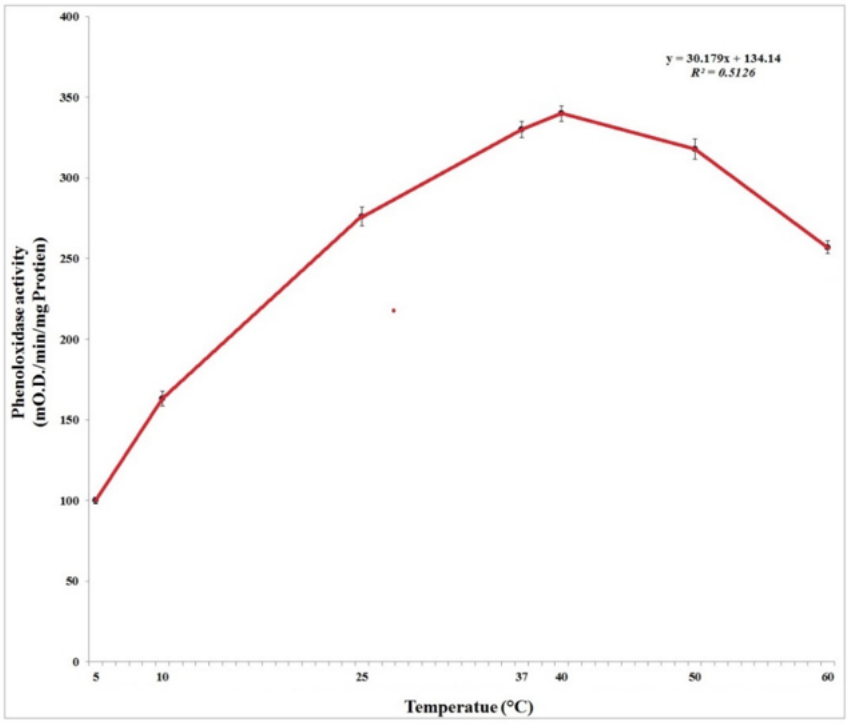

Fig. 5: Effect of reaction temperatures on activity of PO in the 7th larval instar of *R. ferrugineus*. Each point represents the mean of 3 determinations (means±SE).

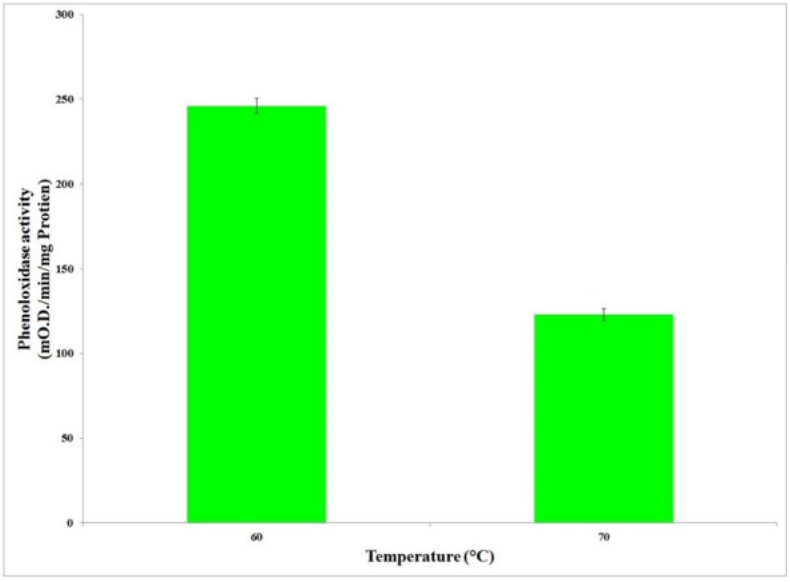

Fig. 6: Effect of the thermostability on of PO activity in *R. ferrugineus*. Each point represents the mean of 3 determinations (means±SE).

Michaelis-Menten Kinetics of Phenoloxidase System:

The reaction kinetics of PO from the 7th instar larvae of the red palm weevil were detected using the Linweaver-Burk plot. (Fig.7). Michaelis-Menten kinetics of PO activity evaluated by constructing Lineweaver Burk double reciprocal plot. Michaelis constant (Km) was, 3.45 $X10^{-4}$ M and maximum velocity (V max) of PO reaction was 344.8 O.D.min-1 mg $protein^{-1}$.

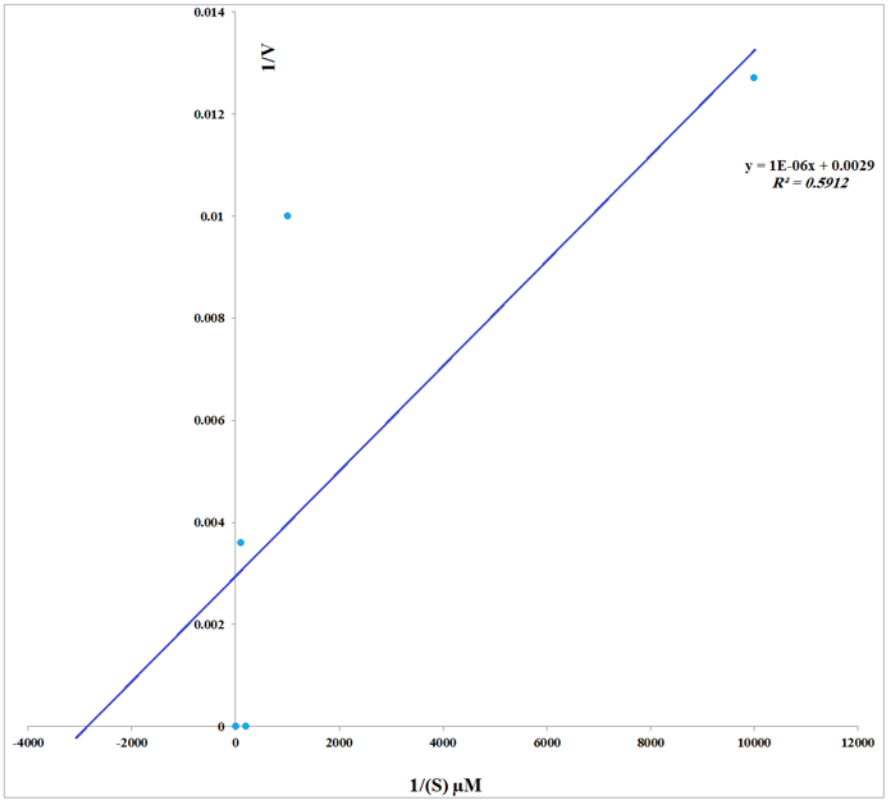

Fig. 7: Double reciprocal (Lineweaver-Burk) plot of 1/V versus 1/(S) for the reaction catalyzed by PO in *R. ferrugineus* at pH 7, 40°C.

According to the previously mentioned findings in the present work, the optimum PH of PO in the 7th larval instar of *R. ferrugineus* was 7. This coincides with, Sharifi *et al.*, (2012) who stated that PO generally in most insects is active in neutral conditions. Similar findings were obtained in different insects as *pieris rapae* (Xue *et al.*, 2006), *Hyphantria cunea* (Sharifi and Ghadamyari, 2014) while the optimum pH was slightly acidic in other insects being 6.5 *Halophora cecropia* (Anderson *et. al.*, 1998) and in *Sarcophaga bullata* (Wang *et al.*, 2004; and Feng *et al.*, 2008), 6.2 in *Parasarcophaga surcoufi* (Ayaad *et al.*, 2001), 6 in *Helicoverpa armigera* (Taleh *et al.*, 2013), *Bombyx mori* (Ashida, 1971) as well as *Eurygaster integriceps* (Zibaee *et al.*, 2011), 5.5 in *Tuta absoluta* (Amin *et al.*, 2013). On the other hand, the optimum PH of PO was alkaline in other insects and reached 8.0 in *Lymantria dispar* (Dunphy 1991) and *Ospheranteria coerulescens* (Gholami *et al.*, 2013) while in *Heliothis virescens* was 9 (Lockey and Orth 1992).

On the other hand, the optimum temperature of PO in the present work was 40°C which is similar to other insects having PO maximum activity equal or closely related to our findings as in *P. rapae* (Xue *et al.*, 2006) and *H. cunea* (Sharifi and Ghadamyari, 2014). In general, the optimum temperature of PO activity ranged PO between 35 and 45°C in most studied insects (Ishaaya, 1970; Lockey and Orth 1992; Zibaee *et al.*, 2011; Amin *et al.*, 2013; Gholami *et al.*, 2013 and Taleh *et al.*, 2013). Amin *et al.*, (2013) concluded that the activity of PO in *T. absoluta* larvae increased gradually after 15°C till reaching the optimum temperature which was 35°C but at 40 and 50°C the enzyme lost its activity by 11.36 and 37.3% respectively, while at 55°C, lost most of its activity by 60.5%. Similarly, in *musca domestica*, PO activity was stable between 0 and 40°C but was unstable in higher than 50 °C and at 60°C PO lost 80% of its activity (Takuji *et al.*, 1986). In contrast, *Apis mellifera* pupae, the optimal

temperature of PO at 20°C (Zufelato *et al.*, 2004).

Phenoloxidase in the present work seems to be highly thermostable as it can withstand high temperatures and still works efficiently. The relatively high optimum temperature and high thermostability of this enzyme may enable it to correspond with the local inflammation at the wound site (Winka *et al.*, 2016). Generally, survival parameters such as the type of pathogens and ambient temperatures play a key role in variation of PO optimal pH and temperature among insects (Liu *et al.*, 2006).

The kinetic parameters of PO in the present study were calculated via Lineweaver–Burk plots. The Michaelis-Menten kinetic constant (K_m) of PO in the present study is 3.45 $X10^{-4}$ M which is relatively higher than *T. absoluta* which was 12.98 $X10^{-6}$ M (Amin et al., 2013) and 0.17 $X10^{-3}$ M in *A. mellifera* (Zufelato *et al.*, 2004) but lower than most insects as being 4.3 $X10^{-4}$ M in *P. hertipes*, 3.93 $X10^{-3}$ M in *Musca domestica* (Wang *et al.*, 2004), 2.25 $X10^{-3}$ M in *H. virescens* (Lockey and Orth, 1992), 10 $X10^{-3}$ M in *E. integriceps* (Zibaee *et al.*, 2011) and 88.61 $X10^{-3}$ M in *O. coerulescens* (Gholami et al., 2013). In general, different values of Km may be attributed to the substrate-binding pocket which may affect substrate catalysis by PO in different insects (Feng et al., 2008). This may indicate that PO in *R. ferrugineus* is relatively ale to hydrolyze its substrate efficiently, even at very low concentrations, and has a high affinity to Catechol.

Since PO is considered as key enzyme of moulting (Gillespie *et al.*, 1997; Sugumaran, 2002). The present study is offering the first step in PO kinetic characterization for the first time in the red palm weevil, *R. ferrugineus* paving the way for further studies to provide clues for enhancing the control of this serious pest via disrupting or inhibiting such vital enzyme as alternative solution rather than traditional pesticides.

Conclusion

Phenoloxidase efficiently catalyze catechol and this may probably facilitate its study as one of innate defence reactions in future for other insects. Reaction rate of PO in the 7^{th} larval instar of *R. ferrugineus* was optimal at 10^{-1} N catechol, neutral medium (pH 7) and 40 ^{0}C. Phenoloxidase kept most of its activity when incubated at 60 C for 15 min before reaction initiation.

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