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## Properties of Phenoloxidases from the Tomato Leafminer, *Tuta absoluta* (Meyrick)

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**Abstract:** The kinetic properties of phenoloxidases system from the fourth larval instar of the tomato leafminer, *Tuta absoluta* (Meyrick) were studied to detect the optimum condition for phenoloxidases-catalyzed reaction. The results of phenoloxidases activity towards catechol at different pH values revealed that the optimum pH was 5.5. The phenoloxidases activity increased gradually from 15 to 35°C and began to decline that phenoloxidases lost 60.5% of their activity at 55°C. A period of only 2 min for the tomato leafminer phenoloxidases was found to fit well within the linear part of the enzyme activity curve. On the other hand, the oxidation was directly proportional to the substrate concentration up to  $10^{-4}$  M, where the peak was reached. Using Lineweaver-Burk plot, it was found that  $K_m$  (Michaelis constant) was  $12.98 \times 10^{-6}$  M, while  $V_{max}$  (maximum velocity of the reaction) was 0.862 O.D. units/min/mg proteins. The relatively small  $K_m$  value indicates that phenoloxidases can hydrolyze catechol efficiently, even at very low concentration. The significance of results and effect of organic solvents on phenoloxidases reaction were discussed. It could be concluded that 1 mL of the reaction mixture consists of 5 µg sample protein and 0.1 mM catechol in 0.1 M phosphate buffer (pH 5.5) at 25°C for 2 min, represents the optimum condition for *T. absoluta* phenoloxidases system activity.

**Key words:** Insecta, *Tuta absoluta*, phenoloxidase, kinetic properties

### INTRODUCTION

The tomato leafminer *Tuta absoluta* (Meyrick) is one of the most important pests of tomato crops (De Medeiros *et al.*, 2005).

Many literatures were cited and deal with control of *T. absoluta* out breaks (Antonio *et al.*, 2011). However, studies concerned with the pest enzyme systems such that of phenoloxidase are relatively few.

The phenoloxidase systems, which in insects leads to sclerotization and tanning of the cuticle and acts as a defensive mechanism against pathogens has been studied in several species (Ishaaya, 1972; Duvic and Brehelin, 1998; Mullen *et al.*, 2004; Shelby and Poham, 2006; Amin and Azazy, 2008; Dorrah, 2009). The phenoloxidases system consists of two types of phenoloxidases: Ortho-diphenoloxidase (tyrosinase, phenoloxidase, catecholoxidase, EC 1.10.3.1) and laccases (EC 1.10.3.2). The former enzymes are very sensitive towards inhibitors, such as thiourea and able to oxidize o-diphenols to o-quinones and being inactive towards p-diphenols. The laccases will oxidize both o-and p-diphenols and they are insensitive towards thioureas (Andersen, 1989).

Reviews about *T. absoluta* phenoloxidase system, to the best of our knowledge, is lacking, so this report aimed to study some kinetic properties of phenoloxidases in *T. absoluta* larvae.

### MATERIALS AND METHODS

**Insects:** *T. absoluta* larvae were a laboratory strain maintained in plant protection research institute, Dokki, Giza, Egypt. When the insects were brought into the analysis laboratory as 4th larval instar, they were frozen at -20°C until used for biochemical assay. Storage for 1 month was possible without significant loss of the phenoloxidase activity.

**Chemicals:** The substrate; catechol and bovine serum albumin were purchased from sigma chemical company (St.Louis). Methanol, ethanol and acetone were from Fluka chemie Gmb (Switzerland). Chemicals used for preparing buffers were purchased from local companies and were of high quality.

**Apparatus:** Insects were homogenized for biochemical analyses in a chilled glass Teflon tissue homogenizer (ST-2 Mechanic-preczyina, Poland).

Double beam ultraviolet/visible spectrophotometer (spectronic 1201, Milton Royco., USA) was used to measure absorbance of colored substances or metabolic compounds.

**Preparation of insects for analysis:** The larvae were homogenized in distilled water (50 mg 1 mL<sup>-1</sup>).

Homogenates were centrifuged at 8000 r.p.m for 15 min at 5°C in a refrigerated centrifuge. The deposits were discarded and the supernatants were kept in a deep freezer till use.

**Kinetic properties of phenoloxidases system:** Phenoloxidase activity was determined with modification of the method described by Ishaaya (1971). The reaction mixture consisted of 0.5 mL phosphate buffer (0.1 M), 200 µL of catechol solutions as substrate and 200 µL of the sample. Effect of pH, substrate concentration and reaction temperature was determined to detect the optimum conditions of the reaction.

Prior to the initiation of the reaction, the substrate and other ingredients were separately incubated at the optimum temperature of the reaction. The absorbance of phenoloxidase activity was recorded for 10 min at 1 min interval against sample blank as the zero adjustment at 405 nm. Phenoloxidase activity was expressed as ΔO.D. units×10<sup>3</sup> min<sup>-1</sup>mg protein<sup>-1</sup>. Total protein of the sample was determined. in each sample by the method of Bradford (1976) using bovine serum albumin as standard.

**Activation and inhibition of phenoloxidases:** The effect of various organic solvents such as methanol, ethanol and acetone (Absolute, HPLC grade) on phenoloxidase activity was determined. This to elucidate some characteristic properties of the tomato leafminer phenoloxidase and to detect some potent inhibitors of this system.

Serial concentration of the solvents were prepared, then 200 µL of the tested solvent were added to 200 µL of the sample and incubated for 5 min at the optimum temperature of the reaction. The reaction was initiated by adding the substrate solution and preceded at the optimum conditions which were found experimentally. The results were compared to reaction mixture containing 200 µL of Δ H<sub>2</sub>O instead of solvent solution.

**Data analysis:** All obtained values were pooled from Triplicate. Using costat statistical software (Cohort software, Brekley), means and standard deviation were obtained and data in Table 1 were analysed by completely randomized one way ANOVA. The means were separated using the Duncan's multiple range test (p<0.01).

## RESULTS AND DISCUSSION

**Optimum condition for phenoloxidase activity:** Effect of pH, temperature, time and substrate concentration on *T. absoluta* phenoloxidase system-catalysed reactions was determined to detect some kinetic properties of such enzymes. Therefore, optimum conditions were determined for each factor separately, all other factors being at the optimum.

The phenoloxidases system activity towards catechol was studied at seven pH values ranging from 4-8 (Fig. 1) The enzyme activity increased sharply between pH 5 and 5.5 (optimal pH 5) and began to decline reaching to the lowest value at pH 8.

Effect of temperature on the oxidation of catechol by phenoloxidase was studied at temperatures ranged between 20 and 55°C (Fig. 2). The activity increased gradually from 15°C to the optimal temperature; 35°C at 40 and 50°C, the activity decreased by 11.63 and 37.3%, respectively, as compared to that of the optimal temperature. This indicates that the enzymes is somewhat thermostable. However, it loses its most activity at 55°C where the activity decreased by 60.5% less than the activity at 35°C.

Table 1: Activation and inhibition of phenoloxidase system from *T. absoluta* larvae

Solvent in the reaction mixture (%)	Phenoloxidase activity (Δ O.D 405×10 <sup>3</sup> min mg <sup>-1</sup> protein)			
	Methanol	Ethanol	Acetone	Control
0	-	-	-	700±30 <sup>e</sup>
2.5	800±25 <sup>b</sup>	688±18 <sup>c</sup>	635±5 <sup>d</sup>	-
5	840±19 <sup>a</sup>	644±25 <sup>d</sup>	574±13 <sup>e</sup>	-
10	518±13 <sup>f</sup>	570±15 <sup>e</sup>	518±10 <sup>f</sup>	-
20	420±8 <sup>b</sup>	378±7 <sup>g</sup>	469±8 <sup>e</sup>	-

Data are presented as the Mean±SD, Values with different letter are significantly different at p<0.01 using Duncan's multiple rang text

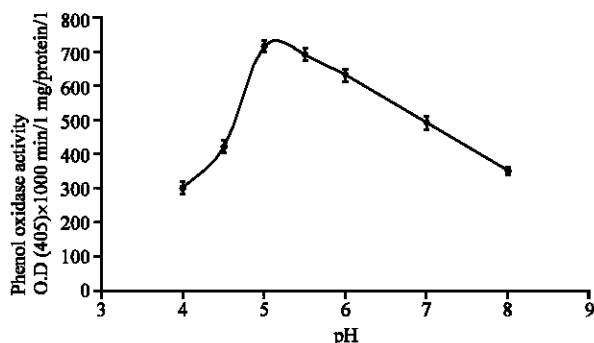


Fig. 1: Effect of pH of reaction on phenoloxidase activity in *T. absoluta* larvae, reaction time was 2 min at 35°C, Each point represents the mean of three determinations vertical bars indicate standard deviation of the mean

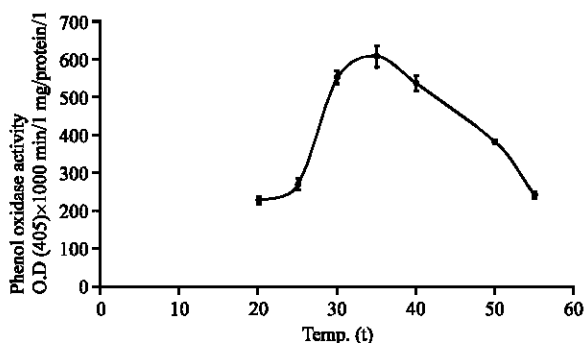


Fig. 2: Effect of temperature of reaction on phenoloxidase activity in *T. absoluta* larvae, Reaction time was 2 min at pH 5.5, Each point represents the mean of three determinations vertical bars indicate standard deviation of the mean

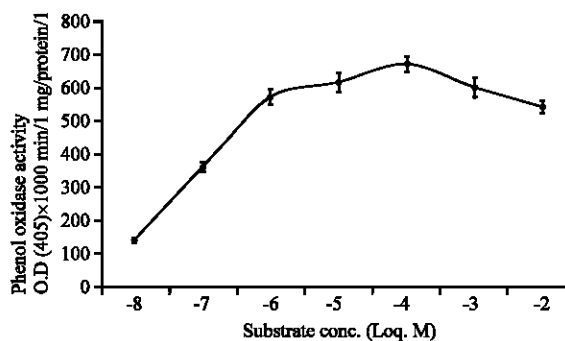


Fig. 4: Effect of substrate concentration of oxidation of catechol by phenoloxidase from *T. absoluta* larvae, Each point represents the mean of three determinations vertical bars indicate standard deviation of the mean

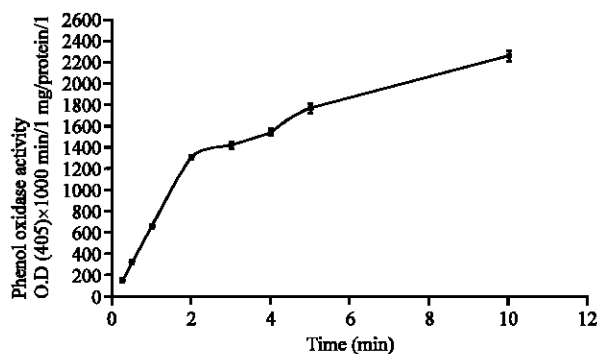


Fig. 3: Reaction time of phenoloxidase activity in *T. absoluta* larvae, Temperature of the reaction was 35°C, pH was 5.5, Each point represents the mean of three determinations vertical bars indicate standard deviation of the mean

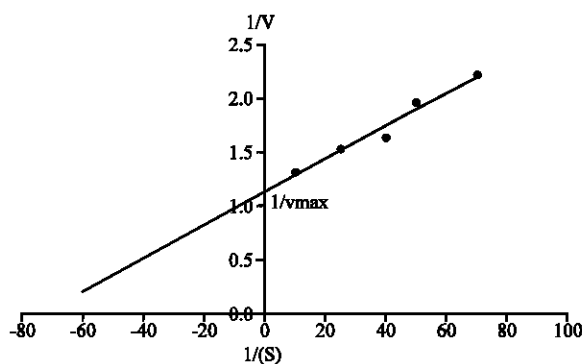


Fig. 5: Double reciprocal (Lineweaver-Burk) plot of  $1/V$  versus  $1/(S)$  for reaction catalyzed by phenoloxidases for *T. absoluta* larvae at pH 5.5, 35°C

Ten minutes were allowed for phenoloxidase reaction to determine the suitable time allowed for phenoloxidase reaction (Fig. 3).

A period of only 2 min for tomato leafminer phenoloxidase activity was found to fit well within the linear part of the enzyme activity curve.

Effect of substrate concentration on the phenoloxidase activity was studied by measuring the activity at seven concentrations ranged between  $3.6 \times 10^{-8}$  and  $10^{-2}$  M (Fig. 4). The oxidation had linear dependence on the substrate concentration up to  $10^{-4}$ , where the peak is reached. The reaction rate decreased as the concentration of the substrate was further increased, indicating substrate inhibition (Bell and Bell, 1988).

**Activation and inhibition of phenoloxidase system:** Some investigators have shown that various organic solvents

such as acetone, ethanol or methanol can induce phenol oxidase activity (Preston and Taybr, 1970).

Table 1 indicate that enzyme pretreatment with methanol strongly enhanced phenoloxidase activity. The efficiency of activation depends on the methanol concentration in the reaction mixture. An enzyme treatment with 5% methanol has the highest reaction rate of about 1.34 folds of the untreated enzyme (control).

Inhibition of phenol oxidase activity occurred when methanol concentration was above 5%, reaching to 40% decrease in activity when the concentration was 20%. Ethanol or acetone showed no stimulatory effect at any of the concentration tested. A significant inhibition of enzymatic activity was obtained with ethanol at concentrations above 5% and of acetone above 2.5%. However, ethanol caused more inhibition than acetone when the concentration reached to 20%.

**Michaelis-menten kinetics of phenoloxidase system:** The kinetics of the phenoloxidases from the tomato leafminer, *T. absoluta* larvae were detected. Maximum velocity of the reaction ( $V_{max}$ ) and substrate concentration ( $K_m$ ) where the velocity of the reaction is one half of maximum velocity were determined using Lineweaver-Burk plot (Fig. 5) When the linear reciprocal plot is extrapolated, it intersects the negative portion of the abscissa at -77 mM, which equal to  $1/k_m$ . Thus,  $k_m$  of reaction catalysed by phenoloxidases from *T. absoluta* larvae was  $12.98 \times 10^{-6}$  M and  $V_{max}$  was 0.862 O.D. units/min/mg proteins.

The optimal temperature (35°C) of *T. absoluta* phenoloxidase activity is usual, as compared with those in other insect. A temperature of 40°C for phenol oxidase obtained from the California red scale and of about 37°C for enzyme activity from the Florida red scale, was found to be the optimum for enzymatic activity (Ishaaya, 1971). Takuji *et al.* (1986) found that latent phenoloxidase from *Musca domestica* was stable at temperature between 0 and 40°C. However, it was fairly unstable at temperatures higher than 50°C and lost 80% of its activity at 60°C. In the present study, the larval phenoloxidases lost 60.5% of its activity as compared to that at the optimal temperature. In comparison to other enzymes such as esterases, phenoloxidases considered to be less thermostable. Zhu and Brindley (1990) found that in periplaneta Americana and Lymantria dispar, the optimal temperature range 50-55°C has been reported for esterases. Moreover, a temperature up to 100°C for 30 min without losing any esterolytic activity was found in *Triatoma infestans* (De Malkenson *et al.*, 1984).

Study on effect of pH on phenoloxidase activity showed that the optimal pH of tomato leafminer phenoloxidases was 5.5. This means that *T. absoluta* phenoloxidase tend to be activated in acidic medium.

Many of phenoloxidases of other insect species had a slight acidic or neutral pH, for example the optimal pH of *Hyalophora cecropia* equals to 6.5 (Andersson *et al.*, 1998); *Pieris rapae*, pH7 (Xue *et al.*, 2006); *Parasarcophaga surcoufi*, pH 6.2 and 6.6 (Ayaad *et al.*, 2001). However, there are some insects which have phenoloxidases that act in acidic pH such as California and Florida red scales, pH 5.5 (Ishaaya, 1971).

Some investigators have shown that various organic solvents, can activate in some cases insect phenoloxidases. Inactivation of *Calliphora* phenoloxidase by alcohols or some proteolytic enzymes such as subtilisin has been reported previously (Cottrell, 1962). The present study showed that methanol up to 5% in the reaction mixture activated phenol oxidases from tomato leafminer. It was suggested that phenoloxidase activation by some solvents results from aggregation of subunits which form the active enzyme (Mitchell and Webber, 1965). In addition to solvents,

acids such as kojic acid might act as a specific inhibitor of phenoloxidase (Shelby and Poham, 2006). The inhibition of phenoloxidases is of a significant importance since it might lead to suppression of insect immunity. Amin and Azazy (2008) found that inhibition of the red palm weevil phenoloxidase by phenylthiourea led the weevil to be susceptible to infection by nematodes.

The Michaelis-menten kinetic constant ( $K_m$ ) of the phenoloxidases from *T. absoluta* larvae is relatively low ( $12.98 \times 10^{-6}$ ), compared with those from other insect species: for example,  $K_m$  of *parasarcophaga* hertipes larvae is  $4.3 \times 10^{-4}$  M (Dorrah, 2009). The small  $K_m$  of tomato leafminer phenoloxidases indicates that they can hydrolyze catechol efficiently, even at very low concentration.

## CONCLUSION

Finally, 1 mL of the reaction mixture consists of about 5 µg sample protein and 0.1 mM catechol in 0.1 M phosphate buffer (pH 5.5) at 25°C for 2 min, represents the optimum condition for *T. absoluta* phenoloxidases system activity.

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