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Purification and characterization of phenoloxidase from immunized haemolymph of *Schistocerca gregaria*

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Phenoloxidase (PO) is a key factor in insect immunity. On invasion of microorganisms and pathogens, prophenoloxidase (PPO) changes to its active form, PO. The present study has been conducted to purify and characterize the PO from the haemolymph of desert locust, *Schistocerca gregaria* (Forskal) following activation of immune system by invasion of bacteria, *Bacillus thuringiensis kurstaki* (*Bt*). PO is purified by a combination of ammonium sulfate precipitation, blue sepharose CL-6B and phenyl sepharose CL-4B chromatography yielded a 209.97-fold purity and 54.75% recovery of activity. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) reveals that the molecular weight of the purified PO is 70.154 kDa. The purified PO is characterized in terms of its biochemical and enzymatic properties by using L-DOPA as a specific substrate. Ca^{2+} and Cu^{2+} significantly stimulated PO activity when compared with other metals. The PO reaction was strongly inhibited by phenylthiourea and thiourea, moderately inhibited by ethylene diamine tetractic acid (EDTA) and poorly inhibited by ethylene glycol tetraacetic acid (EGTA) and diethyl dithiocarbamate (DTC). Inhibition of PO showed excellent recovery ability by addition of Ca^{2+} on EGTA-inhibited enzyme. Therefore, PO is most probably a kind of tyrosinase-type Ca^{2+}-containing metalloenzyme. The content of Ca^{2+} is higher than other trace metal elements. The reactive intermediates yielded by PO with its specific substrate L-DOPA had a broad-spectrum bactericidal activity against Gram +ve bacteria (*Bacillus cereus* and *Staphylococcus aureus*) with a greater degree more than Gram-ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). From the present study, PO from *S. gregaria* is most probably a tyrosinase-type calcium-containing mono-phenoloxidase, which functions not only as a catalytic enzyme in melanin production in locusts, but perhaps also as a humoral factor in host defense via melaninization as in other insects.

Key words: *Schistocerca gregaria*, phenoloxidase, purification.

INTRODUCTION

The desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) represents a relatively important group of plant-feeding insects. They have strong immune responses against bacteria, as previously shown by

(Meshrif and Barakat, 2002; Barakat et al., 2002; Mo'men et al., 2010). The high interest in biological control means of controlling insect pests intensifies the need for investigating the response of insect to disease organisms. The haemolymph offer a readily accessible criterion of this response. The last and more important line of defense is the internal defense system that is comprised of cellular components and humoral mechanisms that offer a very effective protection against invading microorganisms and co-operatively interact to destroy non-self-elements. The cellular defense by haemocytes takes place immediately after contact with the foreign invader (Carton and Nappi, 1997). Humoral mechanisms that are essentially concerned with the ability of insect to recognize and dispose self from non-self, and involve several physiologically active substances, normally present in the native haemolymph or synthesized after natural infections (Boman and Hultmark, 1987). These substances appear in the haemolymph within a few hours after infection and display a broad spectrum of antimicrobial activity. Phenoloxidase (PO) is one of the most important enzymes that involved in the innate immune system of invertebrates. PO is synthesized as an inactive zymogen, prophenoloxidase (PPO) which can be activated by specific proteolysis (Cerenius and Söderhäll, 2004). When insects are infected by microorganisms, PPO activation elicits by microbial cell surface components, such as, lipopolysaccharide (LPS), peptidoglycans, β-1,3-glucose (Mo'men et al., 2012), the activities of the haemocytic enzymes, including phenoloxidase are enhanced during the challenge course.

However, due to the instability and rapid loss of the activity of this enzyme during the purification, more attention is paid to the investigation of PPO. So far, PPO is purified and characterized from only a small number of insect species including Lepidopteran, *Hyalophora cecropia* (Andersson et al., 1989) and *Ostrinia furnacalis* (Feng et al., 2008). Dipteran, *Sarcophaga bullata* (Chase et al., 2000), and cockroaches (Durrant et al., 1993). PO activity is investigated in other insects; *Eurygaster integriceps* (Zibaee et al., 2011) and *Hyphantria cunea* (Ajamhassani et al., 2012). Our knowledge of this enzyme (PO) at the protein level is limited. For example, the exact site of synthesis, regulation of PPO, its activating enzymes and inhibitors are still controversial. Although, there have been a number of studies involving various functional aspects of insect PPO, one or more of these aspects as integral parts of cells could be liberated to act on bacteria upon destruction of the cells or a change in the cell's natural environment. Accordingly, the amount of antibacterial activity in the blood of normal insect should be proportional to the amount of cell destruction or to the degree in which the environment was altered.

The present study aims to isolate, purify and characterize the components involved in the PO cascade system, and to clarify more information dealing with the physicochemical properties of phenoloxidase of *S. gregaria*.

MATERIALS AND METHODS

Maintenance of insects

The desert locust, *S. gregaria* (Forskal), was maintained and reared for ten generations at 30 ± 2°C, a photoperiod of 16:8 (light: dark) and relative humidity varied between 60 and 80%, according to methods of Huxham and Lackie (1989). Cages were illuminated with one electric bulb, 100 watt, per cage in winter, and 60 watt in summer. All experiments outlined below were carried out with adults (both sexes), all being within 2 to 4 days after ecdysis.

Source of the bacterial pathogens

The bacterium, *Bacillus thuringiensis kurstaki (Bt)* (3200 IU/mg, AGERIN- wettable powder) was chosen as the pathogen for this study because of its wide use as a biocontrol agent among insects. The bacterium, *B. thuringiensis kurstaki* (*Bt)* were produced by the Agricultural Genetic Engineering Research Institute (AGERI) at the Ministry of Agriculture, Giza, Egypt. Non-Pathogenic strains of Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), were obtained from Department of Microbiology at Ain Shams University.

Mass culturing of the bacterial pathogens

Subcultures from bacterial pathogens were grown aerobically at 28 ± 2°C in nutrient broth tubes for 48 h. To obtain solitary pure colonies; nutrient agar plates were prepared and cultured with inoculates of the grown bacteria in the nutrient broth, using the streaking dilution method. The plates were incubated at 28 ± 2°C for 48 h. After growth, only solitary colonies were selected, cultured on nutrient agar slants and incubated at 28 ± 2°C for 48 h, and then kept in the refrigerator at 4°C until used. These slants were regenerated monthly.

Injection technique and haemolymph collection

In order to induce activation of locust immune system, which leads to the conversion of prophenoloxidase cascade to its active form (phenoloxidase), a stock suspension of a sub-lethal concentration of *Bt* was prepared, 10 μl of this concentration was injected into the haemocoel of the locusts. Insects were injected with a 10 μl Hamilton micro-syringe fitted with a 26-gauge needle according to Miranpuri and Khachatourians (1993). Ten microlitres of the concentration 1.2×10^6 cells/ml of (*Btk*) were used as a sub-lethal concentration to investigate the subsequent experiments according to Mo'men et al. (2010). Injected locusts were removed from the rearing cages, submerged in hot water bath at 60°C for 2 to 5 min; they were allowed to dry on paper towel. The heat-killed insects were amputated at the hind coxa with fine scissors. The haemolymph was obtained with a fine-tipped calibrated glass capillary, which was kept at -20°C until further analyses.

Estimation of the total haemolymph proteins

The total protein concentration in the haemolymph was quantified according to the method described by Bradford (1976).

Phenoloxidase activity assay

In order to measure PO activity, a preliminary assay was set up, in which we recorded the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) spectrophotometrically at 470 nm, according to Aso et al. (1985) with some modifications. A solution of L-DOPA (2 mg/ml) was made in a sodium phosphate buffer (SPB) (0.01 M, pH 5.9). Aliquots (20 µl) of haemolymph were diluted (v/v) in a sodium cacodylate buffer (SCB) (0.01 M sodium cacodylate, 0.25 M sucrose, 0.01 M trisodium citrate), were added to 2 ml of DOPA solution, after which the formation of dopachrome (reddish brown pigments) was recorded each minute for 5 min to make sure that we respected the linear increase of the optical density. In case of control, 20 µl SCB were used. The phenoloxidase activity was expressed as PO unit, where one unit is the amount of enzyme activity required to produce an increase in the absorbance by 0.001 min/mg protein.

Phenoloxidase purification

All purification steps were performed at 4°C in a sodium cacodylate buffer (SCB) unless otherwise noted. Haemolymph (8 ml) was first diluted into 2:1 with the CB. The saturated ammonium sulphate solution was added to haemolymph until reaching a saturation of 40%. The precipitate was spun down by centrifugation for 10 min at 12,000 rpm, and redissolved in 500 µl of 20 mmol/L sodium cacodylate solution (pH 6.5). The protein was dialyzed in 2000 ml CB overnight at 4°C, and then applied to a blue sepharose CL-6B column (1.0 cm × 10 cm) pre-equilibrated with the CB. The column was eluted with an elution buffer (100 mM/L $CaCl_2$, 10 mM/L Na_2CB and pH 6.5) at a flow rate of 1.5 ml/min. The fractions containing PO from three simultaneous blue sepharose CL-6B chromatography were pooled, and concentrated with sucrose, then applied to a phenyl sepharose CL-4B column (0.8 cm × 12 cm) that was equilibrated with the CB buffer. The column was washed with distilled water at a flow rate of 1.5 ml/min until the absorbance of fractions at 280 nm returned to zero. The fractions with PO activity were dried in a Heto FD3 Model Vacuum Cold Dryer. The purified enzyme was stored at − 80°C according to Feng et al. (2008).

Molecular weight estimation

To determine the success of purification scheme we monitor the procedure of each step by performing, one-dimensional gel electrophoresis in vertical polyacrylamide gel; Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). On 10% gels, using SDS-molecular standard mixture of proteins; 205 kDa: 29 kDa (from Sigma) with a 4% stacking gel, at 100 volts for 5 h at room temperature.

Isolation of PO from polyacrylamide gel

After electrophoresis, the protein band of interest must be located in the gel by Side-strip technique. Staining of strips of the gel cut from the side when isolating abundant proteins that are well separated from other bands, was achieved according to Harlow and David (1988).

Polyclonal antibody production

Specific polyclonal antisera against the isolated PO were raised in young rabbit; approximately 800 µg of the purified PO was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites of the rabbit, at intervals of 4 weeks. Eight days after the final booster, blood was collected and serum prepared according to Fergusson (1996). The antisera were aliquot and stored at -70°C.

Conformation of antibody specificity

It was carried out by using Western blotting analysis. This technique, as described by Towbin et al. (1979), depends on placing a sheet of nitrocellulose against the surface of an SDS-PAGE protein fractionation gel, and then a current is applied across the gel, thus causing the protein to move out of the gel onto the nitrocellulose where they bind firmly.

Effect of metal ions on the PO activity

To verify if the activation of the purified PO is influenced by the presence and concentration of metal ions, the activity of PO in the presence of different metal ions; $MgSO_4$, $ZnSO_4$, $MnCl_2$, $CuSO_4$ and $CaCl_2$, was measured, respectively. According to methods of Feng et al. (2008), the purified PO (10 µg) was added to solutions diluted, respectively, with 100 mmol/L $MgSO_4$, 100 mmol/L $ZnSO_4$, 100 mmol/L $MnCl_2$, 100 mmol/L $CuSO_4$, and 100 mmol/L $CaCl_2$ to various concentrations. 40 µl of 0.1 mol/L sodium phosphate buffer (SPB) and 100 µl of 2 mmol/L L-DOPA were added. The mixtures in a final volume of 1 ml were incubated for 30 min at 30°C, and the increase in absorbance at 490 nm after 10 min was continuously monitored for calculation of the PO activity.

Inhibition assay of PO activity

In order to determine the effect of various inhibitors on the activity of the purified PO, different compounds including: thiourea, phenylthiourea, ethylene diamine tetraacetic acid (EDTA), diethyldithiocarbamate (DTC) and ethylene glycol tetraacetic acid (EGTA), were tested for their inhibitory effect according to the method described by Fan et al. (2009).

Recovery effect of PO activity

To verify the metalloenzyme property of the PO, the recovery effects of some metal ions on PO activity were investigated according to the method of Fan et al. (2009). Recovery effect of Ca^{2+} on the activity of ethylene glycol tetraacetic acid (EGTA) pretreated purified PO from S. gregaria haemolymph, measurement and comparison between the enzymatic activity of 10 µl purified PO only, purified PO + 20 mM EGTA, purified PO + 20 mM EGTA + 10 mM Ca^{2+}, purified PO + 20 mM EGTA + 15 mM Ca^{2+} and purified PO + 20 mM EGTA + 20 mM Ca^{2+} were made; and the reaction mixture was measured spectrophotometrically under the same conditions as described above.

Substrate specificity assay of PO

We investigated the substrate specificity of PO purified from the haemolymph of locusts using the method according to Andersen (1980).

Effect of reactive intermediates produced in PO-catalyzed reactions

In order to test the effect of PO-substrate-derived compounds on the growth and survival of bacterial cells: Bacillus cereus, S.

Table 1. Total haemolymph protein concentration and PO activity of unpurified and purified haemolymph of *S. gregaria,* following activation of immune system with *Bt* injection.

| Haemolymph sample | Total protein Concentration (mg/ml) [A] | PO activity (U/mg)[B] | | Recovery (%)[E] | Purification fold [F] |
		Specific activity [C]	Total activity [D]		
Unpurified	80.537± 0.07**	50.163 ± 1.01**	4039.97±3.6*	100	1
Purified step 1	18.304± 2.26*	185.089±0.04*	3387.86±2.1**	83.85	3.68
Purified step 2	5.001± 0.018*	600.03±0.21**	3000.70±0.23**	74.27	11.96
Purified step 3	0.21± 1.45*	10533.01±0.01*	2211.93±2.10*	54.75	209.97
Control	15.03±0.06	0.003± 0.55		---	---

n=3 replicates per test, (Mean ± SE). *Significance ($P < 0.05$). A, Total protein concentration (mg/ml) determined by dye binding method using BSA as standard protein; B, PO activity measured by L-DOPA (2mg/ml) as a substrate at 470 nm, and expressed in unit/mg protein; C, specific activity; (total activity / total protein of each purification step); D,total activity; (The enzyme activity in the volume of fraction used in the assay x the fraction total volume); E, recover percent (yield); (Total activity retained after each purification step/ total activity of unpurified sample) x 100; Recover percent in the unpurified sample is taken to be 100%. F: Purification fold; (Specific activity calculated after each purification step / specific activity of unpurified sample). Step 1: $(NH_4)_2SO_4$, 40% saturation. Step 2: Blue Sepharose CL-6B chromatography. Step 3: Phenyl Sepharose CL-4B chromatography. **Significant ($P<0.01$) in compared with appropriate control.

aureus, E. coli and *P. aeruginosa,* (dependent on their growth rates), the experiment was carried out according to Zhao et al. (2007).

Statistical analysis

Results of susceptibility test were represented graphically as probit-logarithmic regression line. Statistical analysis of data was made by using software: Probit Analysis Program, Version 4.0. All data of the rest experiments were expressed as mean ± standard error (SE) and analyzed by using the SPSS11.5.0 software (SPSS Inc., 2012). The differences between means were analyzed by independent samples *t*-test and one-way ANOVA. The level of significance for each experiment was set at $P < 0.05$ or $P < 0.01$.

RESULTS

Total haemolymph protein concentration

The unpurified haemolymph of *S. gregaria* adult contained 80.537± 0.07 mg/ml protein. The total protein content of purified haemolymph after serial purification steps using ammonium sulphate precipitation $(NH_4)_2SO_4$, 40% saturation followed by affinity chromatography (Blue Sepharose CL-6B chroma-tography and Phenyl Sepharose CL-4B chromatography) were decreased significantly compared with unpurified haemolymph. Data presented and graphically illustrated in Table (1).

Enzyme purification

The PO purification results are shown in Table 1. The purification procedure yielded a total of 0.21 mg PO from a starting sample of 8 ml haemolymph containing about 80.537 mg total protein. The PO was purified 209.97-fold with a 54.75% total recovery of activity. SDS-PAGE of the purified protein revealed a single band with an estimated molecular mass of approximately 70.154 kDa (Figure 1).
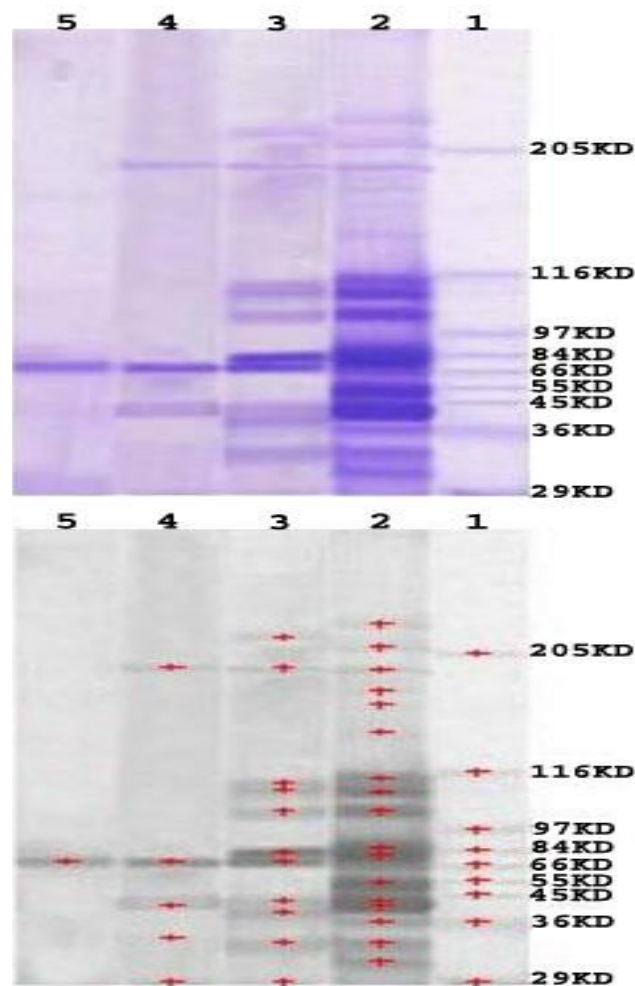

Figure 1. Protein bands of unpurified and purified haemolymph of *S. gregaria* adults determined from SDS-PAGE (10%) method. lane1: Marker of protein; lane 2: haemolymph of *S. gregaria*; lane 3: the precipitation of ammonium sulphate; lane 4: the fractions of Blue Sepharose CL-6B; lane 5: the fractions of Phenyl Sepharose CL-4B.
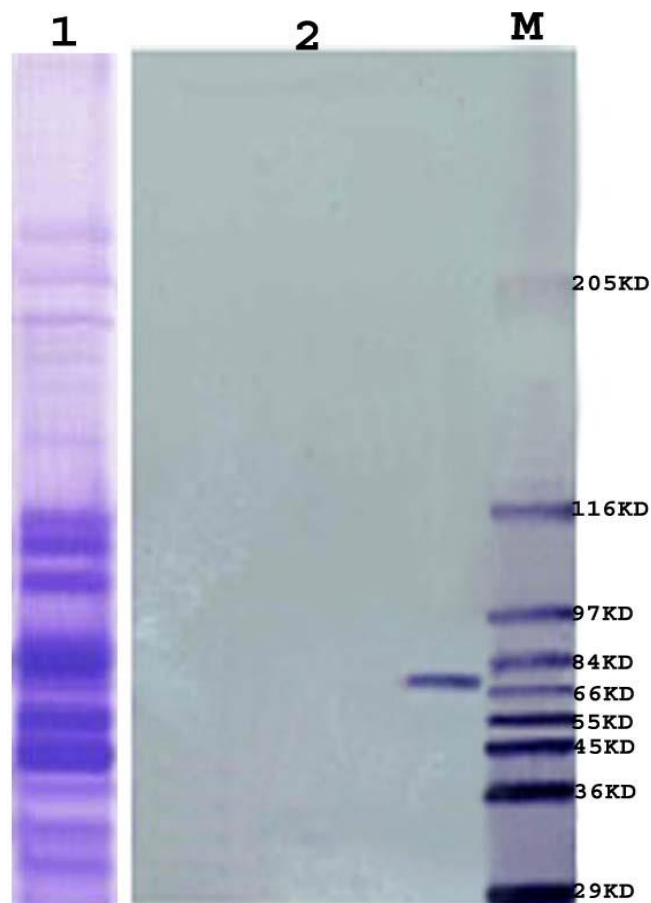
Figure 2. Western blotting analysis of PO purified from the haemolymph of *S. gregaria* adults: Marker of protein; lane 1: haemolymph of *S. gregaria* using SDS-PAGE technique; lane 2: Rabbit antisera against the purified PO.

Confirmation of antibody specificity

Rabbit antisera against the purified PO were obtained. Specific polyclonal antisera against the isolated PO were capable of recognizing one band of approximately 70.154 kDa on SDS-PAGE gels corresponding to that of purified PO band detected from SDS-PAGE (Figure 2).

Effect of metal ions on PO activity

The PO activity was increased when the Ca^{2+}, Mg^{2+} and Cu^{2+} concentration was increased to 15 mM/L. However, when the concentration was increased to 20 mM/L, it became inhibitory. In the presence of Mn^{2+} or Zn^{2+}, the PO activity was increased only by a slight level (Figure 3).

Inhibition assay

The PO activity was completely inhibited by phenylthiourea and thiourea at (20 µM/L), moderately inhibited by ethylendiamine-tetracetic acid (EDTA) and triethylen\tetraminehexaacetic acid (TTHA), poorly inhibited by diethyldithio-carbamate (DTC) (Figure 4).

Recovery effect of Ca^{2+} on the activity of EGTA-pretreated PO purified from activated *S. gregaria* haemolymph

The enzymatic activity of the PO greatly inhibited by 20mM EGTA was restored to its original level by 15 mM Ca^{2+} .This results indicate that PO purified from *S. gregaria* probably is a calcium-containing metalloenzyme (Figure 5).

Enzyme activity and substrate specificity

In addition to L-DOPA, other O-phenols were suitable as substrates for the *S. gregaria* phenoloxidase, while no activity was detected towards the mono-phenol tyrosine (Figure 6).

Effect of PO and L-DOPA derived compounds on the growth of bacterial cells

After PO and L-DOPA had been incubated with bacterial cells, we observed that the bacterial growth was signifycantly reduced (P < 0.05). Bacterial growth was not affected (P > 0.05) after the cells had been treated with the substrate or PO alone (Figure 7). The cell mortality was determined to be higher in the bacterial samples (*B. cereus*, *S. aureus*, *E. coli* and *P. aeruginosa*, respec-tively) treated with PO and L-DOPA. *B. cereus* was most susceptible, while *P. aeruginosa* was most resistant.

DISCUSSION

Several investigators purified and characterized some different insect POs (Durrant et al., 1993; Chase et al., 2000; Fan et al., 2009; Zibaee et al., 2011; Ajamhassani et al., 2012). Three types of POs are reported in insects as follows: laccase type (E.C.1.10.3.2; p-diphenol: O2 oxidoreductase), catechol oxidase type (E.C.1.10.3.1; diphenol: O2 oxidoreductase), and tyrosinase type (E.C.1.14.18.1; monophenol, L-DOPA: O2 oxido-reductase) (Barrett, 1987). The major problem to analyze the PO is the lack of effective tools to identify and quantitate individual PO isoforms. Several investigators use conventional chromatographic method (Chase et al., 2000) instead of immunoaffinity chromatography which is ineffective for purifying PO (Kopácek et al., 1995). In the present study, a combination of ammonium sulfate precipitation, blue sepharose CL-6B chromatography and
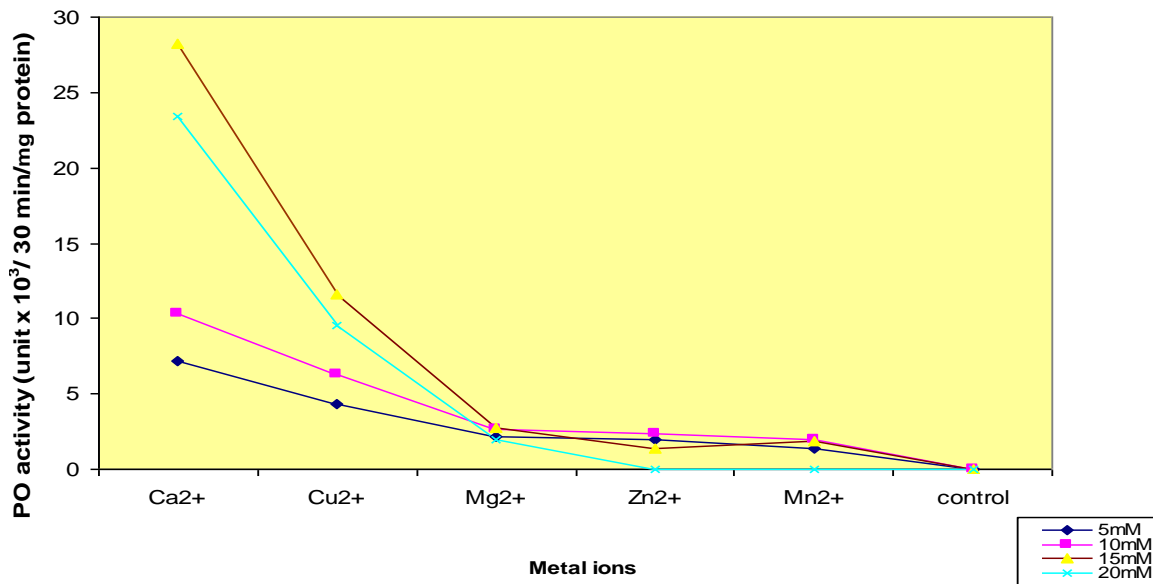
Figure 3. Effect of various metal ions on the activity of PO purified from the activated haemolymph of *S. gregaria* adults.
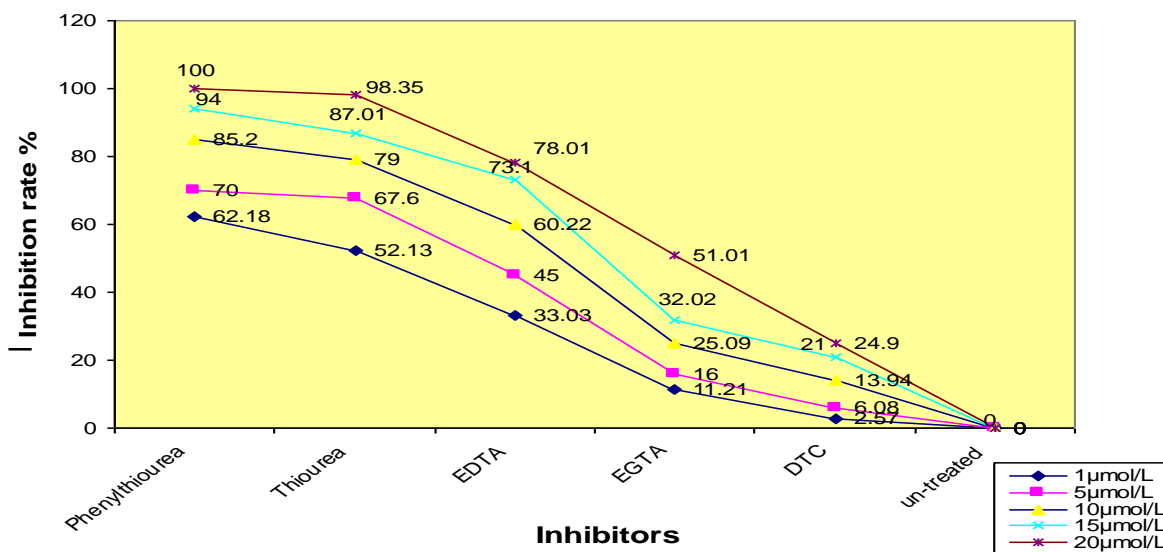

Figure 4. Effect of various inhibitors on the activity of PO purified from the activated haemolymph of *S. gregaria* adults.

phenyl sepharose CL-4B chromatography was employed to purify the PO from the haemolymph of *S. gregaria*. The enzyme estimates as 70.154 KDa by gel filtration in Sepharose and SDS-PAGE. These data are compatible with the purified enzyme from *S. bullata* (Chase et al., 2000), *H. cecropia* (Anderson et al., 1989), *Locusta migratoria* (Cherqui et al., 1996), *E. integriceps* (Zibaee et al., 2011), *H. cunea* (Ajamhassani et al., 2012) and *Helicoverpa armigera* (Goudru et al., 2013), that a single

isoform characterizes from them. Many reports detect different isoforms of PO in several insects, for example, there are two isoforms in *Galleria mellonella* (Kopácek et al., 1995) and *Bombyx mori* (Yasuhara et al., 1995), three isoforms in the fruit fly *Drosophila melanogaster* (Fujimoto et al., 1993), and *Branchiostoma tsingtauense* (Pang et al., 2005), six in the mosquito *Anopheles gambiae* (Müller et al., 1999), The physiological significance of PO isoforms in the above mentioned insects
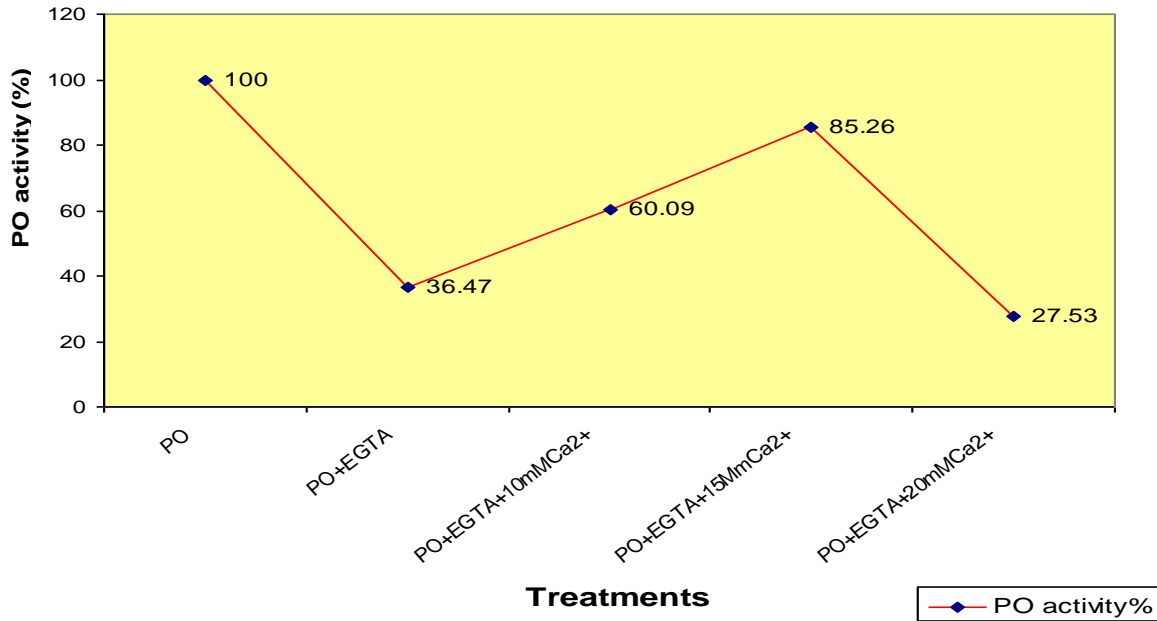
Figure 5. Recovery Effect of Ca^{2+} on the activity of EGTA pre-treated PO purified from the activated haemolymph of *S. gregaria* adults.
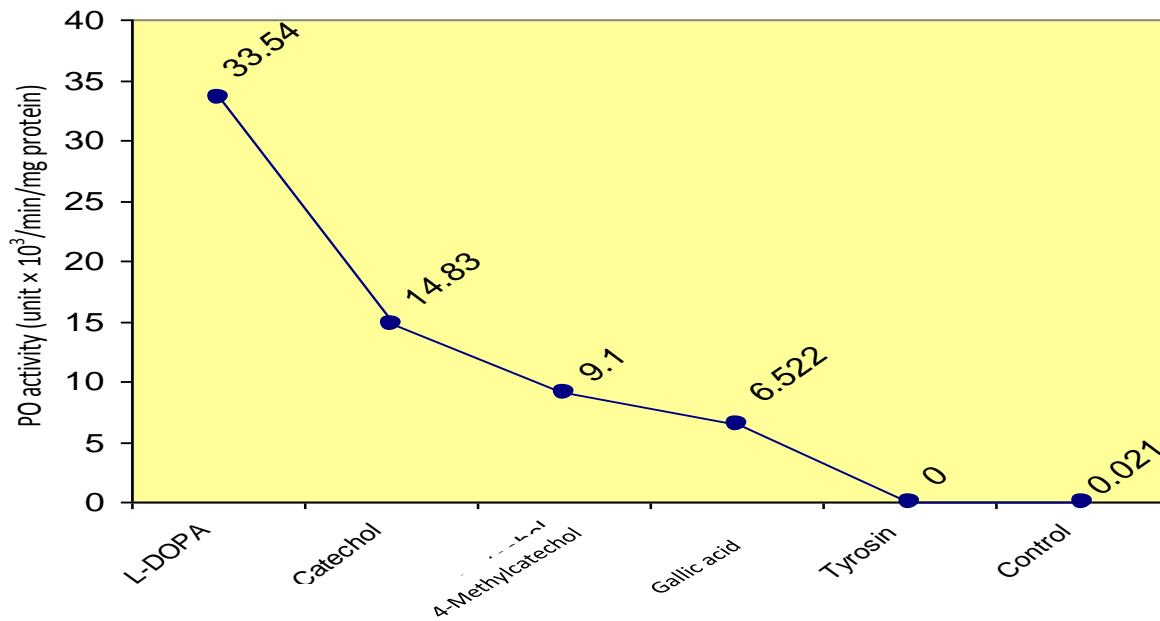

Figure 6. Substrate specificity and the corresponding activity of PO purified from the activated haemolymph of adult *S. gregaria.*

still remains to be studied (Feng et al., 2008). Different substrates can adopt the appropriate conformation to interact with the PO protein. There is no information at this time on the substrate binding pocket in PO. The differences in the substrate binding pockets between the different insects are probably the result of differences in

substrate-protein contact points or differences in the size of the substrate binding pocket reference. In the present study, the PO from *S. gregaria* is capable of oxidizing L-DOPA effectively, but fails to oxidize tyrosine. These results implies that this enzyme is most probably a kind of monophenol, tyrosinase-type *o*-oxidoreductase, not a
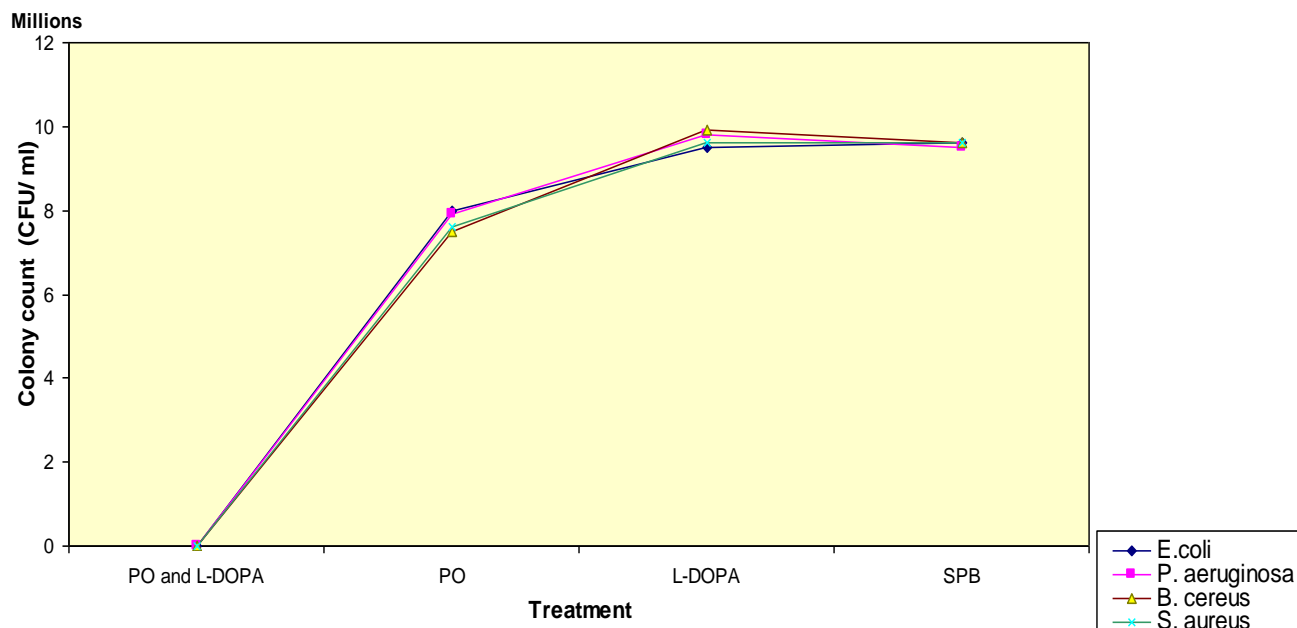
Figure 7. Effect of reactive intermediates (generated *in vitro* by PO purified from the haemolymph of *S. gregaria*) on the growth of Gram +ve and Gram -ve bacterial cells.

laccase-type or catechol oxidase-type enzyme, this is in agreement with the results of Cherqui et al. (1996), Pang et al. (2005) and Asano and Ashida (2001).

Phenoloxidase activity is almost entirely inhibited by phenylthiourea and thiourea. This complete inhibition may be attributed to the influence of phenylthiourea on this process which is caused by its interaction with active sites of PO rather than with intermediate products of DOPA oxidation preventing the subsequent melanin formation. This explanation is in agreement with the results of Ryazanova et al. (2012). Results also indicate that treatment with EGTA nearly showed a similar pattern of inhibition as EDTA, since EDTA is a divalent cation scavenger and EGTA is a specific calcium chelator. Inhibition of PO activity with EDTA, indicates the involvement of divalent cations in the melanin-synthesis pathway, it may resemble other invertebrate POs that contain multiple copper atoms and/or copper binding sites (Aspan and Söderhäll, 1991; Aspan et al., 1995; Nellaiappan and Sugumaran, 1996). In fact, the effects of both EDTA and EGTA may be due to calcium dependency of the POs, as calcium is known to increase activity of several invertebrate POs (Perdomo-Morales et al., 2007), suggesting that the binding of some calcium atoms is necessary in the activating center of *S. gregaria* PO. The result of recovery test show that the enzymatic activity of the purified PO greatly inhibits by 20 mM EGTA, restores to its original level by 15 mM Ca^{2+}. It was concluded that *S. gregaria* PO is most probably a kind of calcium-containing metalloenzyme and different from other insects such as *Heliothis virescens* (Lockey and

Ourth (1992). DTC is a specific chelator for cupper presence in PPO which may explain the poor inhibition of *S. gregaria* PO activity that indicates few cupper atoms exist in the *S. gregaria* PO. This explanation is in accordance with those of Feng et al. (2008) on *O. furnacalis* larvae. Some metal ions can significantly modify the structure of PO (Li et al., 2000) that leads to increase or decrease in the activity of the enzyme, this ability to change conformation in solution might explain how the enzyme enhances its activity. Several metal ions tested with PO of *S. gregaria* showed that PO activity increases significantly when the Ca^{2+} concentration increases to 15 mM/L. However, when the concentration increases to 20 mM/L, it became inhibitory. In the presence of Cu^{2+} the PO activity increases only by a moderate level, while Mg^{2+}, Zn^{2+} and Mn^{2+} show non-significant increase in the PO activity. So, it was concluded that the content of calcium is higher than other trace metal elements. Calcium-mediated PO activity enhancement has been reported for a large number of insects: for example *B. mori* (Ashida et al., 1983), *S. gregaria* (Dularay and Lackie, 1985), *Blaberus craniifer* (Leonard et al., 1985), *L. migratoria* (Brehelin et al., 1989), *Lymantria dispar* and *Galleria mellonella* (Dunphy, 1991). Thus, the knowledge of the binding of trace metal elements to the PO is required to be investigated extensively.

Insect POs, or tyrosinase-type POs, are similar to mammalian tyrosinases with two catalytic activities: the oxygenase activity which hydroxylates monophenols to o-diphenols and the oxidase activity which converts o- diphenols

to quinones (Sugumaran, 2002; Nappi and Christensen, 2005). It has long been known that insects rely heavily on tyrosine metabolism for cuticle hardening and for innate immune responses (Vavricka et al., 2014). It was found that the roles of melanization include anti-bacterial, anti-fungal, anti-viral and anti-parasitic responses. This reaction has a broad-spectrum for all possible agents that can invade insects. This kind of universal killing power seems to stem from its basic mechanism of toxicity (Zhao et al., 2007). Several comprehensive reviews covering the humoral immunity discussed a number of immune proteins that were induced by the injection with bacteria (Gtz and Boman, 1985; Boman and Hultmark, 1987; and Hultmark, 1993). Barakat (1997) indicated that the humoral defense reactions needed to some extent for newly synthesis and release of the antibacterial proteins. Meshrif and Barakat (2002) investigated the appearance of antibacterial substances in the haemolymph of the bacterial-injected insects as well as the uninjected insects, the antibacterial activity needs certain time to appear and integrate with the cellular reactions to produce an effective immune response in this species. Therefore, we decided to test the controversial function of PO directly by measuring possible antimicrobial activity of the reactive compounds produced *in-vitro* by this enzyme. After treating bacteria with the reaction mixtures containing purified *S. gregaria* PO with its specific substrate L-DOPA, the antibacterial effect (growth inhibition of bacterial cells) was observed. These findings established that the reactive intermediates yielded by PO had a broad-spectrum bactericidal activity against bacteria. Gram +ve bacteria (*B. cereus* and *S. aureus*) are more susceptible than Gram-ve bacteria (*E. coli* and *P. aeruginosa*). These results are similar to those of Zhao et al. (2007) who reported the antimicrobial effect of reactive intermediates produced in phenoloxidase-catalyzed reactions after being treated with *Manduca sexta* PO and dopamine, *Bacillus subtilis* ceased to grow.

Cerenius et al. (2010) revealed that an active PO isolated from the freshwater crayfish *Pacifistic leniusculus* exhibited a strong antibacterial effect *in-vitro* on the bacteria Gram -ve whereas, a weaker but still significant effect against Gram +ve. Rowley et al. (2011) investigated the possible role of the PPO system of *L. migratoria* in the killing/inhibition of growth of several species of bacteria, and suggesting that the antimicrobial factor(s) may have been generated by either the PPO cascade or a related enzyme system. The limited data gathered so far seem to indicate that certain bacterial species are more sensitive than other to quinone inter-mediates produced in the melanization cascade. These intermediates may have developed a tolerance to the presence of some bacteria. These results established that PPO activation is an integral component of the insect defense system involving a multitude of enzymes (e.g. proteinases, oxidases, and dopachrome conversion enzyme (DCE), which immobilize and kill invading microorganisms. The nature of these bioactive molecules requires detailed study to characterize the significance of these compounds. From the present study, PO from *S. gregaria* is most probably a tyrosinase-type calcium-containing mono-phenoloxidase, which functions not only as a catalytic enzyme in melanin production in locusts, but perhaps also as a humoral factor in host defense via melaninization as in other insects. To understand the similarities as well as differences in molecular characterization and physiological function among these arthropod POs, it is necessary to conduct more accurate, qualitative and quantitative analyses by cloning and transcriptional or translational detection of PO.

Conflict of interests

The author(s) did not declare any conflict of interest.

Abbreviations: EDTA, Ethylene diamine tetractic acid; **EGTA,** ethylene glycol tetraacetic acid; **DTC,** diethyl dithiocarbamate; **AGERI,** agricultural genetic engineering research institute; **L-DOPA,** L -dihydroxyphenylalanine; **SCB,** sodium cacodylate buffer.

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