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RESEARCH REPORT

# Purification and characterization of phenoloxidase from the hemolymph of *Hyphantria cunea* (Lepidoptera: Arctiidae)

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**Abstract**

Phenoloxidase (PO) is a key factor in insect immunity. On invasion of microorganisms and pathogens, prophenoloxidase changes to its active form to PO. This study investigated purification biochemical properties of PO from the hemolymph of 5$^{th}$ instar larvae of *Hyphantria cunea* (Lepidoptera). The purification fold was determined as 9.67 with a recovery of 0.12 and a specific activity of 23.28 U/mg protein identified. Kinetic parameters of purified PO from the insect *H. cunea* were determined. The Michaelis constant ($K_m$) and the maximal velocity ($V_{max}$) were 4.08 and 12.98 µmol/min/mg protein, respectively. The optimal pH and temperature of the enzyme for oxidation of L-DOPA were 10.0 and 35 °C, respectively. The ions $Zn^{2+}$, $Cu^{2+}$, $K^+$ and $Na^+$ significantly increased the enzyme activity and synthetic inhibitors such as diethyldithiocarbamate (DETC) significantly decreased it. Finally, it was found that purified PO had a molecular mass of 33 kDa. This study demonstrated some PO properties and its inhibitory effects demonstrating that it can be employed as useful methods for developing novel insecticides to replace traditionally used ones.

**Key Words**: purification; phenoloxidase; *Hyphantria cunea*; enzyme property

## Introduction

Insect immunity consists of both humoral and cellular defensive reactions (Gillespie *et al*., 1997; Lavine and Strand, 2002). Cellular immunity includes phagocytosis, encapsulation and nodule formation (Lavine and Strand, 2002). But antimicrobial peptides and the phenoloxidase (PO) system both have major roles in humoral defense. PO is a key factor of insect immunity, having important roles in the processes of coagulation, melanization and wound healing.

In arthropods PO is synthesized as inactive zymogen and prophenoloxidase is then activated in the presence of serine proteinases. This phenomenon occurs when pathogen agents and parasitoids enter the hemocel (Söderhäll and Cerenius, 1998). PO causes modifying tyrosine to form dihydroxyphenylalanine, and they oxidize *o*-diphenols to quinones to form melanin for nodulation and encapsulation. Melanotic encapsulation and nodulation play important roles in innate immune response against large microorganisms (Ling and Yu, 2005). Insects have both types of phenoloxidases, namely laccase-type enzymes (EC 1.10.3.2) and tyrosinase-like enzymes. Laccase-type change oxidize *o*- or *p*-diphenols to quinones, this function is fundamental in sclerotization and tanning of the cuticle (Dittmer *et al*., 2004; Arakane *et al*., 2005). Also, those enzymes in the hemolymph that have tyrosinase-like activity can hydroxylate tyrosine (EC 1.14.18.1) and oxidize *o*-diphenols to quinones (EC 1.10.3.1) (Gorman *et al*., 2007a).

The function of proPO in arthropods' immunity and regulation of its activating system have been discussed in other researches (Ashida and Brey, 1997; Cerenius, 1998; Sugumaran, 2002; Cerenius and Söderhäll, 2004; Kanost *et al*., 2004; Christensen *et al*., 2005; Nappi and Christensen, 2005). PO activity has been investigated in other insects such as *Manduca sexta* (Lepidoptera: Sphingidae), (Hall *et al*., 1995), *Drosophila melanogaster* (Diptera: Drosophilidae) (Sezaki *et al*., 2001), *Apis mellifera* (Hymenoptera: Apidae) (Zufelato *et al*., 2004), *Pieris rapae* (Lepidoptera: Pieridae) (Xue *et al*., 2006), *Heliothis virescens* (Lepidoptera: Noctuidae) (Shelbi *et al*., 2006), *Ostrinia furnacalis* (Lepidoptera: Pyralidae) (Feng *et*

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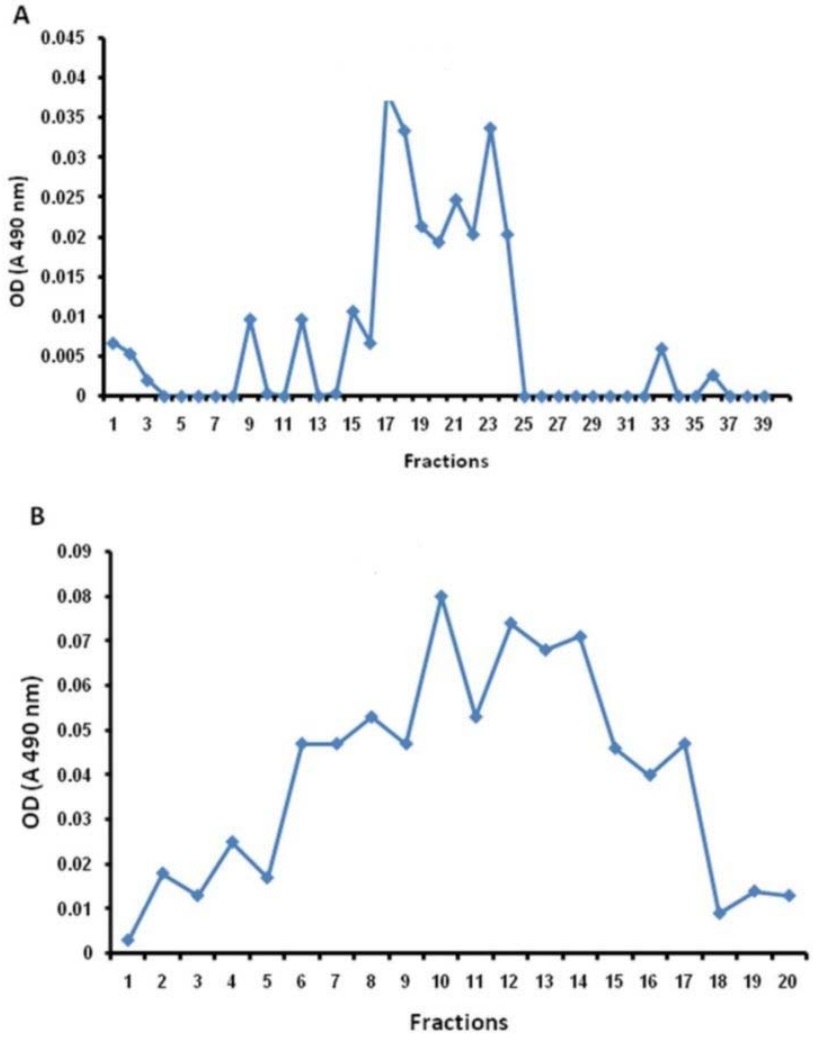


**Fig. 1** Column chromatography of PO from *H. cunea*. Profiles corresponding to 490 nm absorbance and enzymatic activity of collected fractions are shown. A) Sepharyl G-100 gel-filtration of PO after ammonium sulfate (30 % and 70 %) treatment. PO was applied to a Sepharyl G-100 column and eluted with 25 mM Tris-HCl buffer (pH 8). Fractions 17 - 24 contained the highest enzymatic activity on 10 mM L-DOPA and collected for next steps. B) CM-Sepharose ion-exchange chromatography of the gel-filtrated PO from *H. cunea*. Sepharyl G-100 runoff fractions were applied to a CM-Sepharose column and eluted with a linear gradient (1, 3 and 5 M ) NaCl in 25 mM Tris-HCl buffer (pH 8). Fractions 10 - 14 contained the highest enzymatic activity on 10 mM L-DOPA and used for continuing the experiments.

*al*., 2008), and *Eurygaster integriceps* (Hemiptera: Scutelleridae) (Zibaee *et al*., 2011). Several studies have investigated purification and characterization of different insect POs (Durrant *et al*., 1993; Gillespie *et al*., 1997; Chase *et al*., 2000).

*Hyphantria cunea* or fall web worm (Lepidoptera: Arctiidae) is a polyphagous insect found throughout the world. It was first reported in Iran in 2002 in the Caspian forests (Guilan province, northern Iran). A large population of *H. cunea* has been established during the last few years and the insect is one of the most harmful pests in the region. This insect has a broad range of hosts from different forest trees, fruit trees and ornamentals to annual crops and weeds. The preferred hosts include morus, maple, platanus, oak, poplar, elm, fagus, willow and alder (Yarmand *et al*., 2009). There is a need for an advanced program to keep this pest under control. The use of *Bacillus thuringiensis* has been worked out as an entomopathogenic agent decreasing the population of larvae of *H. cunea* in the field. Furthermore, pilot experiments have shown that *Beauveria bassiana* can affect its survival and longevity (unpublished data). Ajamhassani and colleagues evaluated decreases in total hemocytes, nodulation and PO activity of *H. cunea* against different isolations of the fungus *B. bassiana* (unpublished data). But there appears to be very little known research on PO in this species. Therefore, it is essential to obtain more information on the physicochemical properties of *H. cunea* PO. The purpose of this study was to purify PO from the plasma of *H. cunea*, and to examine its biochemical properties.

**Table 1** Purification of the PO from the hemolymph of *H. cunea*.

| Purification steps | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Recovery (%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 0.36825397 | 0.153667 | 2.40688889 | 100 | 1 |
| Ammonium sulfate 30 % | 0.26243386 | 0.017667 | 15.4372941 | 72.22 | 6.413796 |
| Ammonium sulfate 70 % | 0.23915344 | 0.016333 | 14.9470625 | 63.88 | 6.210117 |
| Sepharyl G-100 | 0.08465608 | 0.004 | 21.164 | 0.23 | 8.793094 |
| CM-Sepharose | 0.04656085 | 0.002 | 23.2805 | 0.12 | 9.672445 |

## Material and Methods

*Insects*

Eggs of *Hyphantria cunea* Drury were collected from the forests of Guilan province and reared on mulberry leaves in laboratory conditions (26 ± 1 °C, 80 % RH and 14 h light:10 h dark). Two-day-old 5$^{th}$ instar larvae were used in this study.

*Collection of hemolymph*

Hemolymph samples (10 larvae, 200 µl hemolymph) were collected from severed third prolegs of fifth instar larvae. The hemolymph was immediately diluted with an anticoagulant solution (0.01 M ethylenediamine tetraacetic acid, 0.1 M glucose, 0.062 M NaCl, and 0.026 M citric acid, pH 4.6) (Azambuja *et al*., 1991). The hemolymph was diluted with a phosphate buffer (pH 7, 10 mM).

*PO preparation*

The collected hemolymph (35 µl of hemolymph and 15 µl of anticoagulant solution) was mixed with the phosphate buffer and centrifuged at 6,000 g for 30 min. Supernatant was mixed with 25 g ammonium sulfate and centrifuged at 6000 g for 30 min. Pellets were mixed with Tris HCl 25 m M (pH 8). This crude extract was used to assess PO. Samples were pre-incubated with the buffer (Tris-HCl, pH 7) at 30 °C for 30 min before the addition of 50 ml of 10 mM aqueous solution of substrate L-dihydroxyphenylalanine (L-DOPA). The mixture was incubated for 5 min at 30 °C and PO activity (One unit of PO activity represents the amount of enzyme required to produce an increase in $OD_{490}$ of 0.01 per min) was measured in a spectrophotometer at 490 nm (Dularay and Lackie, 1985). Assays were done in triplicates.

*Purification of PO*

Purification of the PO extracted from *H. cunea* hemolymph was done according to a three-step procedure described by Pang *et al.* (2005) at 30 ˚C: a) *Ammonium sulfate treatment*. The samples were subjected to ammonium sulfate precipitation using concentrations of 30 % and 70 %. Then two different levels of ammonium sulfate treatments were collected by centrifugation at 10,000 g and the pellets obtained in each treatment were suspended in a minimal volume of 100 mM Tris-HCl, pH 8.0. To change samples from the first ammonium sulfate precipitation to the second one, the 30 % pellet was discarded, the supernatant precipitated with 70 % ammonium sulfate and the pellet was used for the next step; b) *Sepharyl G-100 gel filtration*. The ammonium sulfate fractions were subjected to gel filtration using a Sepharyl G-100 column (2 cm×100 cm) equilibrated with 25 mM Tris-HCl pH 8.0 containing 0.05 % (v/v) Triton X-100. Fractions of 5 ml were collected at a flow rate of 20 ml/h with the same buffer. Protein content and PO activity were measured and fractions showing PO activities were pooled; c) *CM-Sepharose separation*. Fractions with

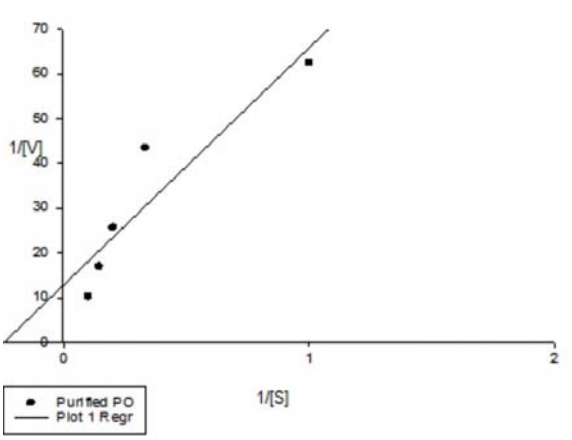


**Fig. 2** Double reciprocal plot to show the kinetic parameters of the purified PO from the hemolyph of *H. cunea* L-DOPA (10 mM) was used as substrate. ($1/V_{max}$ = intercept on the $1/V_0$ ordinate, $-1/K_m$ = intercept on the negative side of the 1/[S] abscissa).

PO activity were applied to a CM-Sepharose column (3 cm×30 cm) equilibrated with 25 mM Tris-HCl buffer pH 6.0. After washing the column with the same buffer, bound proteins were eluted with a linear gradient of NaCl (1, 3 and 5 M) in the equilibrating buffer. Fractions (5 ml each were collected at a flow rate of 1.0 ml/min. Fractions with PO activities were pooled and stored at -20 °C for further analysis.

*Kinetic parameters ($V_{max}$ and $K_m$) of PO*

To measure the kinetic parameters of PO different concentrations of L-DOPA (1, 3, 5, 7 and 10 mM) were mixed with 20 µl of enzyme solution and read at 490 nm. Michaelis constant ($K_m$) and maximal velocity ($V_{max}$) was estimated by Sigmaplot software version 11 and the results of $K_m$ and $V_{max}$ were the means ± SE of three replicates (n = 3) for each concentration.

*Effects of pH and temperature on the enzyme activity*

The effects of temperature and pH on PO activity were examined using 10 mM solution of L-DOPA as a substrate. Optimal pH was determined using 25 mM Tris-HCl buffer at a range of 4 - 12. The effect of temperature on PO activity was determined by incubating the reaction mixture at temperatures of 20, 25, 30, 35, 40, 50 and 60 °C for 30 min (Liu *et al*., 2006), followed by measurements of activity.

*Effects of ions and enzyme inhibitors on protease activity*

The effect of various ions (0.5, 3 and 5 mM) on enzyme activity was investigated using $CaCl_2$, CuCl, $ZnSO_4$, $MgCl_2$, KCl and NaCl. The activity of the enzyme in the absence of added ions was considered as 100 %. The effect of enzyme inhibitors on PO activity was studied using ethylenediaminetetraacetic acid (EDTA, 10mM), diethyldithiocarbamate, N, N, N′, N′-tetraacetic acid (EGTA, 10 mM), sodiumdodecylsulfate (SDS, 10 mM), phenylthiourea (10 mM), diethyldithiocarbamate (DETC, 10 mM) and phenylmethylsulfonyl fluoride (PMSF, 10 mM). The purified enzyme was pre-incubated with the inhibitors for 30 min at 35 °C and pH 8, and enzyme activity was determined with L-DOPA as a substrate. In all experiments, the activity of the enzyme without the addition of inhibitors was considered as 100 percent.

*Polyacrylamide gel electrophoresis*

SDS polyacrylamide gel electrophoresis (denaturing SDS-PAGE) was used to determine the purity and molecular mass of the enzyme as described by Laemmli (1970) using a 4 % (w/v) stacking gel and a 10 % (w/v) separating gel. The molecular mass of the enzyme was estimated using the following standards: β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35.5 kDa), restricting endonuclease Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa) an lysozyme (14.4 kDa). After SDS-PAGE, proteins on the polyacrylamide gel were stained with 0.2 % Coomassie brilliant blue R-250.

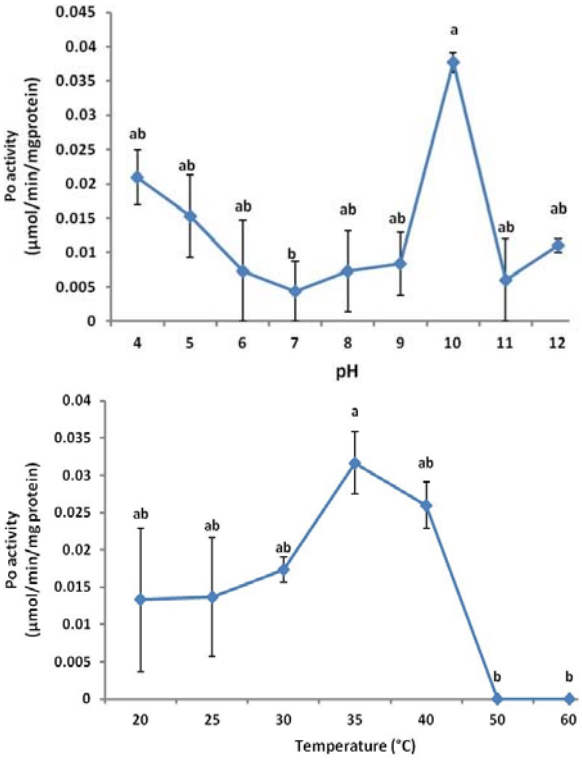


**Fig. 3** Effect of pH (A) and temperature (B) on the activity of the hemolymph-derived PO in *H. cunea*. Different letters show significant differences among values (Tukey's test, $p < 0.05$).

*Protein determination*

Protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad) as a standard.

*Statistical analysis*

Data were compared by the one-way analysis of variance (ANOVA) followed by Tukey's studentisized test and significant differences were considered at $p < 0.05$ (SAS, 1997). Significant differences were marked in figures and tables.

**Results**

*Purification of PO*

PO from the hemolymph of *H. cunea* was purified and the results are presented in Fig. 1 and Table 1. In the first step, samples were precipitated with ammonium sulfate in concentrations of 30 % and 70 %. In the second step, the 70 % ammonium sulfate fraction was eluted by gel filtration using Sephadex G-100 column followed by ion exchange chromatography on CM-Sepharose column. About 10 ml of the ammonium sulfate-precipitated PO was loaded onto a Sepharyl G-100 gel-filtration column (Fig. 1A). Fractions 17 - 24 with high enzymatic activities using 10 mM L-DOPA were collected and pooled. The pooled fractions contained 0.004 mg/mL protein and the total enzymatic activity was

**Table 2** Effect of different compounds (ions as activators and organic molecules as inhibitors) on the PO activity of *H. cunea*.

| Compounds | Concentration (mM) | Percentage of PO activity (%) |
|---|---|---|
| Control | - | 100 |
| $Cu^{+}$ | 0.5 | 106.19 |
| | 3 | 129.64 |
| | 5 | 202.65* |
| $Zn^{2+}$ | 0.5 | 215.04* |
| | 3 | 217.69* |
| | 5 | 273.45* |
| $Ca^{2+}$ | 0.5 | 27.43 |
| | 3 | 26.54 |
| | 5 | 9.73 |
| $Mg^{2+}$ | 0.5 | 24.77 |
| | 3 | 12.38 |
| | 5 | 12.38 |
| $K^{+}$ | 0.5 | 29.20 |
| | 3 | 123 |
| | 5 | 199.11* |
| $Na^{+}$ | 0.5 | 38.93 |
| | 3 | 42.47 |
| | 5 | 136.28 |
| EDTA | 10 | 72.94 |
| EGTA | 10 | 83.52 |
| DETC | 10 | 2.35* |
| PMSF | 10 | 22.35* |
| Phenylthiourea | 10 | 56.47* |

The activity of the PO was determined by incubating the enzyme in the presence of various compounds for 30 min at 30 °C and pH 8. Asterisks show the significant differences among treatments and control (Tukey's test, $p < 0.05$).

0.23 U. The pooled fractions from Sepharyl G-100 gel-filtration were loaded onto an ion-exchange column (Fig. 1B). Fractions 10-14 with high enzymatic activities were eluted at a NaCl concentrations 1,3 and 5 M, and were pooled. The 3 ml pooled fraction had a protein concentration of 0.002 mg/ml and a total enzymatic activity of 0.046 U. The final purification step achieved 9.67-fold purity with a recovery of 0.12 % and a specific activity of 23.28 U/mg proteins (Table 1). Kinetic analyses using Lineweaver-Burk plots showed that $V_{max}$ of purified PO was 12.98 U/mg proteins with a Km of 10 mM (Fig. 2).

*Effect of pH and temperature on PO activity*

The effect of pH on PO activity of *H. cunea* hemolymph was analyzed by pH range of 4 - 12. Activity reached a maximum value at pH 10 with L-DOPA substrate. The activity decreased more than 50 % when other pH levels were used. At 35 ºC, PO showed maximum activity. At 10 ºC below this maximum value, *i.e.*, at 25 and 20 ºC, the activity decreased to approximately 60 %. There was a decrease recorded of approximately 100% when the level of activity was above that of the temperature tested (Fig. 3).

*Effect of ions and inhibitors*

The effects of various ions (0.5, 3 and 5 mM) on activity of the purified enzyme were studied at pH 10 and 35 °C (Table 2). Experiments were carried out at 25 - 30 °C because this was considered suitable for growth and development of *H. cunea* in the forest. Different concentrations of $Cu^{+}$ and $Zn^{2+}$ increased PO activity, the highest effect correlated to high concentrations of these ions (202.65 % and 273.65 %) respectively, compared with the control (Table 2). Also, the highest concentration of $K^{+}$ and $Na^{+}$ had more effect on activity of the purified enzyme (199.11 % and 136.28 %) whereas additions of $Mg^{2+}$ and $Ca^{+}$ decreased the activity of the enzyme (Table 2), in other experiments, respectively.

Enzyme activity was measured in the presence of different enzyme inhibitors (Table 2). Results indicated that some inhibitors including DETC (10 mM), PMSF (10 mM) and phenylthiourea (10 mM) decreased enzyme activity to 2.35 %, 22.35 % and 56,47%, respectively (Table 2).

*SDS-PAGE*

SDS-PAGE showed a single major protein band at molecular mass 33 kDa compared with a large smear of proteins in the crude extract (Fig. 4).

## Discussion

In this study we characterized a PO enzyme in the hemolymph of *H. cunea*. The native enzyme was estimated as 33 kDa by gel filtration in Sepharose and SDS-PAGE. These data are compatible with the purified enzyme from *Sarcophaga bullata* (Chase *et al*., 2000), *Hyalophora cecropia* (Anderson *et al*., 1989), *Locusta migratoria* (Cherqui *et al*., 1998) and *E. integriceps* (Zibaee *et al*., 2011) that a single isoform has been characterized from them. It has been reported that different isoforms of PO have been detected in several insects. For example, there are two isoforms in *G. mellonella* (Kopácek *et al*., 1995) and *Bombyx mori* (Yasuhara *et al*., 1995), six in the mosquito *Anopheles gambiae* (Müller *et al*., 1999) and three in the fruit fly *Drosophila melanogaster* (Fujimoto *et al*., 1993). However, it is essential to have more detailed investigations on the structure of PO. The physiological significance of PO isoforms in the above mentioned insects still remains to be studied (Feng *et al*., 2008).

Analysis by Lineweaver-Burk plots identified the kinetic parameters of the enzyme, $V_{max}$ and $K_m$ using L-DOPA as the substrate measured 12.98 µmol/min/mg protein and 4.08 mM, respectively. $K_m$ was higher than that of other insects, such as the *H. virescens* with 2.25 mmol (Lockey and Ourth, 1992), *Spodoptera littoralis* with 1.35 mmol (Lee and Anstee, 1995), *A. mellifera* with 0.17 mmol (Zufelato *et al*., 2004), housefly (*Musca domestica*) pupae with 3.93 mmol, blowfly (*Sarcophaga bullata*) pupae with 1.54 mmol (Wang *et al*., 2004), *P. rapae* with 0.8 mmol (Xue *et al*., 2006), *O. furnacalis* with 0.92 mmol (Feng *et al.*, 2008) but lower than *E. integriceps* with10 mmol (Zibaee *et al*., 2011).

Catalytic efficiency was calculated by $V_{max}$ and $K_m$ in the presence of L-DOPA. Although L-DOPA has traditionally been used as a substrate for characterization of POs from arthropods in general (Durrant *et al*.,1993; Kopácek *et al*.,1995; Lee and Anstee, 1995; Brivio *et al*.,1996; Cherqui *et al*., 1996), data in this study showed a low affinity of *H. cunea* PO for L-DOPA . The PO binding affinity was significantly affected by the nature of the active part of PO. However, differences in substrate-protein contact points or differences in the size of the substrate-binding pocket can affect PO binding affinity in different insects (Feng *et al*., 2008). It would appear that sometimes insect PO prefers other substrates.

The pH that permitted the higher *H. cunea* PO activity, *i.e.*, 10, was different from those obtained for this enzyme from the other species that were studied, for example, pH 7.5 for *G. mellonella* (Dunphy, 1991), 7.4 and 7.5 for larval and pupal PO from *M. domestica* (Hara*, et al*., 1993), pH 7.0 - 7.5 for *S. littoralis* (Lee and Anstee, 1995), pH 6.5 for *A. mellifera* (Zufelato *et al*., 2004), pH 7.0 for *P. rapae* (Xue *et al*., 2006) and pH 6.0 for *E. integriceps* (Zibaee *et al*., 2011). However, some reported optimum pH values were higher, such as pH 8.0 for PO from *L. dispar* (Dunphy, 1991), and pH 9.0 for this activity in *H. virescense* (Lockey and Orth, 1992), suggesting distinct properties for PO from different sources.

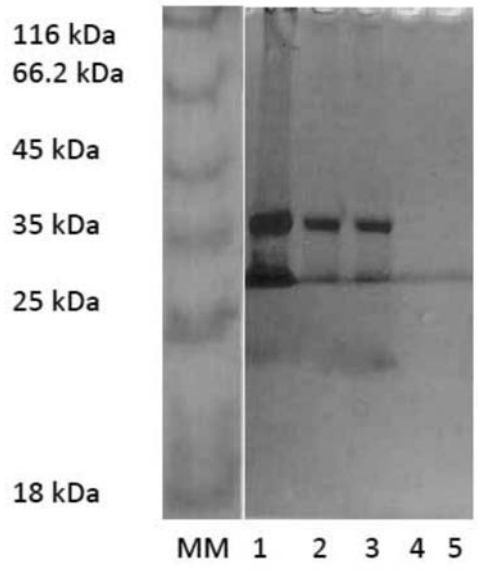


**Fig. 4** SDS-PAGE of the purified PO from *H. cunea*. Left to right; MM: molecular mass markers, 1: crude extract, 2: ammonium sulphate 30 %, 3: Ammonium sulphate 70 %, 4: Sepharyl G-100 chromatography and 5: CM-Sepharose ion exchange chromatography.

The optimal temperature was 35 - 40 ºC for the L-DOPA catalysis reaction with PO activity. For several species, PO showed maximum activity at 30 - 45 º C. such as *L. migratoria* (30 – 35 ºC) (Cerqui *et al*., 1996), *E. integriceps* (30 - 35 ºC) (Zibaee *et al*., 2011) and *H. virescens* (45 ºC) (Lockey and Orth, 1992). Also, the present result showed that PO activity reached approximately 0 when the temperature was 50 - 60º C as the purified PO was not extremely heat stable and in most cases was partially or totally destroyed after short exposure to temperature above 50 or 60 ºC.

Several metal ions (0.5, 3.5 mM) were tested with PO of *H. cunea* showing that all concentrations of $Zn^{2+}$ and $Cu^{2+}$ and high concentration (5 mM) of $K^+$ and $Na^+$ increased PO activity, whereas $Mg^{2+}$ and $Ca^{2+}$ decreased enzyme activity. $Zn^{2+}$ increased PO activity 2 - 2.5 fold. Copper is the center of the PO structure and causes high activity in the presence of $Cu^{2+}$. This phenomenon was verified using specific chelating agents of $Cu^{2+}$ and $Zn^{2+}$. This result is similar to other studies (Anderson *et al*., 1989; Feng *et al*., 2008; Zibaee *et al.*, 2011). $Ca^{2+}$ -modulating PO activity enhancement has been reported for a large number of insects, *e.g*., *B. mori* (Ashida *et al*., 1983), *Schistocerca gregaria* (Dularay and Lackie, 1985), *Blaberus craniifer* (Leonard *et al*., 1985), *L. migratoria* (Brehelin *et al*., 1989), *L. dispar* and *G. mellonella* (Dunphy, 1991), but in this research $Ca^{2+}$ decreased PO activity. Lockey and Orth (1992) reported $Ca^{2+}$ was not required for PO activity in *H. virescens*. Some metal ions can significantly modify the structure of PO (Li *et al*., 2000) that leads to increased or decreased

PO activity. Also, the structure of PO could be reversed by salt concentration. However, in the presence of high concentrations of $K^+$ and $Na^+$ PO activity increased in *D. melanogaster* (Sezaki *et al.*, 2001) that was also likely in this study.

Among inhibitors, DETC significantly decreased PO activity ($p < 0.05$), DETC is a specific chelator for copper. Inhibition of *H. cunea* PO by DTC was similar to that found in $PO_S$ of *H. virescens* (Lockey and Qurth, 1992), *Aedes aegypti*, *Anopheles quadrimaculatus* (Nayar and Bradley, 1994) and other arthropods such as *Limulus polyphemus* (Nellaiappan and Sugumaran, 1996), *O. furnacalis* (Feng *et al*., 2008) and *E. integriceps* (Zibaee *et al*., 2011). PMSF as an inhibitor affects PO activity in *H. cunea*. It also abolished PO activity in venom and plasma of parasitoid *Nasonia vitripenni* (Abt *et al*., 2007). In contrast, however EDTA and phenylthiourea are important inhibitors of PO activity in insects, it showed no inhibitory effect on *H. cunea*. It seems these differences are correlated to the molecular structure of the enzyme.

PO has crucial roles in insect development and immunity (Xue *et al.*, 2006; Gorman *et al.*, 2007a). Hence it should be possible to control insect pests by inhibiting this enzyme. This could provide a basic tool for the development of new insecticides to replace others being used that are environmentally threatening. For example Xue *et al.* (2006) found that 4-hexylresorcinol and 4-dodecylresorcinol were effective PO inhibitors. This research may be considered as a basis for future investigation on inhibitors in these insects.

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