Entomological Research

Entomological Research **43** (2013) 55–62

RESEARCH PAPER

Purification and characterization of prophenoloxidase from cotton bollworm, *Helicoverpa armigera*

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Received 11 June 2012;
accepted 29 October 2012.

doi: 10.1111/1748-5967.12002

Abstract

Phenoloxidases are oxidative enzymes, which play an important role in both cell mediated and humoral immunity. Purification and biochemical characterization of prophenoloxidase from cotton bollworm, *Helicoverpa armigera* (Hübner) were carried out to study its biochemical properties. Prophenoloxidase consists of a single polypeptide chain with a relative molecular weight of 85 kDa as determined by SDS–PAGE, MALDI–TOF MS and LC–ESI MS. After the final step, the enzyme showed 71.7 fold of purification with a recovery of 49.2%. Purified prophenoloxidase showed high specific activity and homology with phenoloxidase subunit-1 of *Bombyx mori* and the conserved regions of copper binding (B) site of phenoloxidase. Purified prophenoloxidase has pH optima of 6.8 and has high catalytic efficiency towards the dopamine as a substrate in comparison to catechol and L-Dopa. The PO activity was strongly inhibited by phenylthiourea, thiourea, dithiothreitol and kojic acid.

Key words: copper binding B site, *Helicoverpa armigera*, Kojic acid, prophenoloxidase.

Abbreviations: Bm PO, *Bombyx mori phenoloxidase;* CPC, cetylpyridinium chloride; ESI MS, electron spray ionization mass spectrometry; Ha PO, *Helicoverpa armigera phenoloxidase;* MALDI – TOF MS, matrix assisted laser desorption ionization Time of flight mass spectrometry; LC-ESI MS, liquid chromatography electron spray ionization mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PO, phenoloxidase.

Introduction

The cotton bollworm *Helicoverpa armigera* (Hubner) is a major polyphagous pest of pulses, cereals, cotton and vegetables. It feeds on more than 182 species of crops and the annual loss in cotton and pulses alone has been estimated at US$300–500 million annually (King 1994). Phenoloxidase (PO) (1.14.18.1) catalyzes the oxidation of monophenols to o-diphenols and o-diphenols to the corresponding quinones (Sugumaran 1998). PO is usually synthesized and exists as an inactive proenzyme, prophenoloxidase (PPO). PPO is an integral part of the innate immune system of insects. PPO can be readily activated through limited proteolysis by a specific serine type of proteinase in response to cell wall components such as peptidoglycans, β-1, 3 glucan and lipopolysaccharides (Kopacek *et al*. 1995; Yasuhara *et al*. 1995; Jiang & Kanost 1997). PO is associated uniquely with many physiologically important biochemical processes in insects such as sclerotization, encapsulation, melanization and wound healing, and hence, it plays a major role in insect immunity and development. PO-generated quinones participate and serve as substrate for quinine methide sclerotization and cross linking with cuticular structural proteins and chitin resulting in the hardening of the cuticle (Sugumaran 1998). PPO present in the hemocytes is released and activated to produce melanin pigment for deposition on the intruder

(Ashida *et al*. 1990). Thus, encapsulation and melanization of foreign organisms are considered to be a defense reaction (Gillespie *et al*. 1997). Similarly, during wound healing of the insect cuticle, PO causes massive deposition of melanin polymer at the wound site to prevent continuous loss of hemolymph and block the entry of opportunistically invading microorganisms or pathogens (Ashida 1971). The unique roles played by PO in insect physiology and biochemistry certainly need to be studied, however, numerous problems such as instability, stickiness, spontaneous activation, rapid loss of activity during purification, browning of sample and self-inactivation have prevented detailed characterization of insect POs (Sugumaran & Kanost 1993). Taking advantage of the fact that PO is present in the zymogen form, two isoforms of PPO have been successfully purified and characterized in *Bombyx mori* (Ashida 1971; Yasuhara *et al*. 1995), *Manduca sexta* (Hall *et al*. 1995; Jiang *et al*. 1997), *Galleria mellonella* (Kopacek *et al*. 1995), *Ostrinia furnacalis* (Feng *et al*. 2008) and *Musca domestica* (Hara *et al*. 1993). Only one isoform, however, has been characterized from *Heliothis virescens* (Lockey & Ourth 1992), *Blaberus discoidalis* (Durrant *et al*. 1993), *Hyalophora cercropia* (Andersson *et al*. 1989) and *Locusta migratoria* (Cherqui *et al*. 1996). In anopheline cell line, Müller *et al*. (1999) have characterized six different PPO genes, indicating the presence of six different isozymes.

Helicoverpa armigera has developed high levels of resistance to commonly used pesticides and, therefore, it is important to gain an understanding of the enzymatic system involved in its defence mechanism. Therefore, the present studies were undertaken on purification of PPO from *H. armigera* larvae and its biochemical properties characterized, including its molecular weight. Information on the mechanism of defense reaction mediated by phenoloxidase will be useful to develop newer approaches to control this pest in future.

Materials and methods

Chemicals

Dopamine, catechol, phenylmethane sulfonyl fluoride (PMSF), thiourea, phenylthiourea, Kojic acid and cetylpyridinium chloride (CPC) were purchased from Himedia Chemicals, Mumbai, India. Blue Sepharose CL-6B, Phenyl Sepharose CL-4B were from Sigma Aldrich Chemicals (St Louis, MO, USA). All other chemicals used were of analytical grade from Loba Chemie, CDH and Himedia (Mumbai), India.

Insects

Laboratory-reared larvae of *H. armigera* was supplied by the Mass Production Unit, National Bureau of Agriculturally Important Crops of Insects (NBAII), ICAR, Bangalore, India. The insects were reared continuously on a synthetic diet consisting of chickpea powder, methyl paraben, yeast, multivitamin tablets, and formalin (Ballal 2007).

Enzyme purification

All the operations were carried at 0–5 °C, unless otherwise stated, except for column purification, which was carried out at room temperature. After removal of gut contents, the fifth instar larvae were homogenized in buffer A (50 mM sodium phosphate buffer pH 6.8 and 1 mM PMSF) containing 25% $(NH_4)_2SO_4$. The homogenate was centrifuged at 15 000 g for 20 min and the supernatant was immediately collected and subjected to 60% $(NH_4)_2SO_4$ saturation and proteins precipitated within 30 min were collected by centrifugation at 15 000 g for 15 min. The pellet was dissolved in minimum volume of buffer A and loaded on blue sepharose CL-6B column (1 × 20 cm) pre-equilibrated with buffer A. The column was washed with buffer A at a flow rate of 1.5 ml min^{-1}. Fractions exhibiting PO activity were pooled (15 ml) and saturated with 10% $(NH_4)_2SO_4$ and eventually loaded on to phenyl sepharose CL-4B (0.8 × 12 cm) column equilibrated with buffer A containing 10% $(NH_4)_2SO_4$. After loading and washing the column to remove the unbound proteins, the column was eluted with buffer A without $(NH_4)_2SO_4$ at a flow rate of 1.5 ml min^{-1} until the absorbance of fractions at 280 nm became zero. The active fractions were pooled and used for further biochemical characterization. Protein concentration was determined by (Lowry *et al*. 1951) using bovine serum albumin as a standard protein.

Assay of PO and PPO activity

The PO activity was measured according to the modified method of (Hall *et al*. 1995). Briefly, the reaction mixture (1 ml) consisted of 50 mM phosphate buffer, pH 6.8, 20 µl enzyme and 2 mM dopamine substrate (60 µl). The increase in absorbance due to the oxidation of the substrate was monitored at 475 nm up to 3 min. The PPO assay was carried out after activating the enzyme protein by the addition of 10 µl of 10% CPC for 5 min, and then the assay was carried out as described above. One unit of enzyme activity is defined as the increase in absorbance of 0.001/min.

Electrophoresis and activity staining

Native polyacrylamide gel electrophoresis (PAGE) was carried out as described by the Davis (1964) method. Activity staining of PO was performed using pyrocatechol and p-phenylenediamine (Lamikanra & Watson 2001). After electrophoresis, the gels were incubated in 50 mM sodium

phosphate buffer, pH 6.8, containing 0.1% coupling reagent p-phenylenediamine in the dark for 20 min, followed by the addition of 0.5 M pyrocatechol as a substrate and incubated until brown color bands appeared.

Molecular weight determination

SDS-PAGE

Molecular weight of PPO was determined under denaturing conditions using 8% SDS-PAGE (Laemmli 1970) using standard molecular weight markers in the range of 29–205 kDa (Sigma-Aldrich).

MALDI–TOF MS

To determine the molecular mass of PPO polypeptides, matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI–TOF MS) was employed. Spectrometry was performed on an Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany) by operating it in linear mode. A saturated solution of sinapinic acid matrix was prepared with 50% acetonitrile/H_2O with 0.1% TFA (v/v); equal volume of sample and the matrix (1 µl) were mixed in a centrifuge tube. One microlitre of the solution was spotted on a MALDI plate, and the sample thus mixed was allowed to dry before analysis. The software used for acquiring and analyzing the mass spectra was Flex control and Flex analysis 3.0, respectively.

LC–ESI MS

The intact molecular weight of PPO was determined using HCT ultra PTM discovery system (ETD-II Bruker Daltonics, Germany) with 1100 series HPLC (Agilent) at a flow rate of 0.5 ml/min fitted with a Zorbax 300SB-C_8 column (9.4 × 250 mm). Peptide separation on LC was operated by gradient elution with mobile phase A (water in 0.1% HCOOH) and B (acetonitrile in 0.1% HCOOH). The following gradient profile was applied: 5% B from 0–5 min, 5–40 % B from 5–20 min, and 40–95 % B from 20–45 min.

Mass-spectrometric analysis

A coomassie blue-stained spot was cored from a 1.5 mm thick 1-D SDS-PAGE gel and digested with trypsin. The ingel piece as well as intact purified fraction were collected in a sterile, siliconized microcentrifuge tube and submitted for MALDI-MS and LC-ESI MS analysis at Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India. The MALDI-MS of the tryptic peptide mixture (*m*/*z*) values were searched among the entries in the MASCOT database. The partial peptide sequences were compared with those of the protein from a MASCOT and BLAST similarity search (http://www.ncbi.nlm.nih.gov/blast) to obtain a homology.

Enzyme kinetics and substrate specificity

Biochemical characterization requires activation of PPO to PO by incubating with 10 µl of 10% CPC for 5 min, then the effect of pH on the PO activity in oxidation of dopamine was studied using buffers (50 mM) of various pH range sodium acetate for pH (5.0–6.0), sodium phosphate for pH (6.0–7.0), Tris-HCl for pH (7.0–8.0), glycine-NaOH for pH (8.0–9.0). Substrate specificity for PO was checked using diphenols such as dopamine, catechol and L-dopa. For different substrates, the quantity of enzyme (20 µl) in the reaction mixture was determined to obtain a linear activity curve. The Michaelis–Menten constant (Km), maximum velocity (Vmax) and specificity (Vmax/Km) for different substrates were determined by plotting the activity data as a function of substrate concentration according to the Lineweaver-Burk method (Lineweaver & Burk 1934).

Inhibition assay

Different compounds such as thiourea, phenylthiourea, N-bromosuccinimide, sodium azide, kojic acid, O-phenanthroline and dithiothreitol were tested for their inhibitory effect on the activity of purified PPO. A solution containing 20 µl of purified PPO was incubated for 5 min with 10 µl of 10% CPC to obtain the active enzyme PO. Then 940 µl of the buffer containing the enzyme solution with 1 mM concentration of inhibitors was incubated for 10 min at 30°C. After adding the substrate, the reaction was triggered and absorbance was recorded for 3 min under standard assay conditions. The inhibitor constant (Ki) for kojic acid was determined by keeping both the enzyme and substrate concentrations constant, and subsequently increasing the inhibitor concentrations.

Results

PPO purification

The PPO was purified from the fifth-instar larvae using a combination of ammonium sulphate precipitation, blue sepharose CL-6B chromatography and phenyl sepharose CL-4B chromatography. The extraction buffer containing 25 % ammonium sulphate was used to remove the low molecular weight proteins that interfere with the activation of PPO. The highest PPO activity was found in the 60 % ammonium sulphate fraction. Figure 1 shows the typical elution profile of PPO through blue sepharose CL-6B column, in which the PPO was eluted as unbound protein. Among the fractions collected, fraction numbers 25–32 having PPO activity, were pooled. The final purification step achieved the specific activity of 28 181 Umg^{-1} protein, with a purification fold of about 71.7 and a total recovery of 49.2% (Table 1).

Molecular weight determination

Under denaturing conditions on SDS PAGE, purified PPO moved as a single band corresponding to 85 kDa when compared with the standard molecular weight markers (Fig. 2a, lane 1&2). The PO activity staining was observed in the native PAGE and compared with the corresponding CBB staining (Fig. 2b, lane 1&2). The molecular weight of purified PPO was also verified with MALDI–TOF MS (Fig. 3). Figure 3 shows the extended region without the doubly charge, representing an intact molecular weight of about 84 309 Da, with an intensity of between 750 and 1000 (a.u.). The charge distribution profile of LC-ESI MS spectra of the protein which eluted at 45.4–46.0 min as shown in (Fig. S1). The deconvoluted mass of the protein is 84 688 Da, showing 100% of abundance (Fig. S1).

MS analysis of HaPPO

The peptides mass fingerprinting (PMF) was analyzed using mascot database search using other metazoa as taxonomic entry. The matched sequences were subjected to NCBI blast; putative conserved domains were detected, which belonged

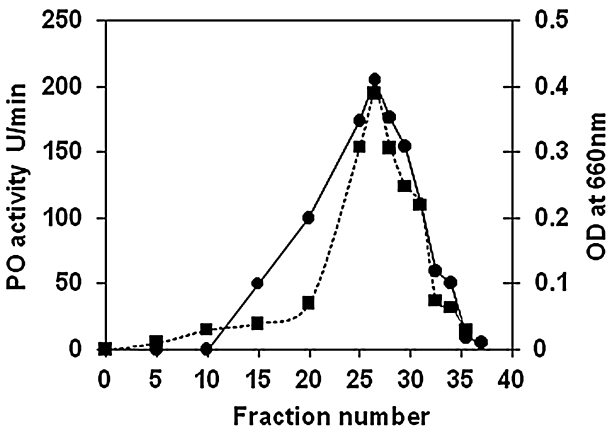

Figure 1 Elution profile of phenoloxidase from *Helicoverpa armigera* through Blue Sepharose CL–6B column chromatography (•) protein (A_{660nm}); (■) phenoloxidase activity at 475 nm.

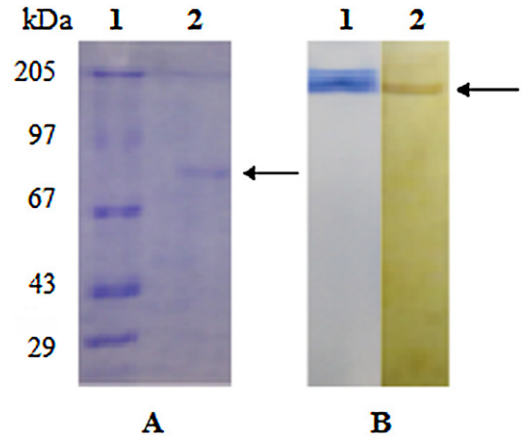

Figure 2 (A) SDS–PAGE of purified PPO from *Helicoverpa armigera*. Lane 1, marker proteins (Myosin 205 kDa, Phosphorylase b 97 kDa, bovine serum albumin 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 29 kDa); lane 2, purified PPO under reducing conditions (8 %). (B) Activity staining of PPO from *H. armigera*. Lane 1, CBB stained purified native band; lane 2, PPO staining.

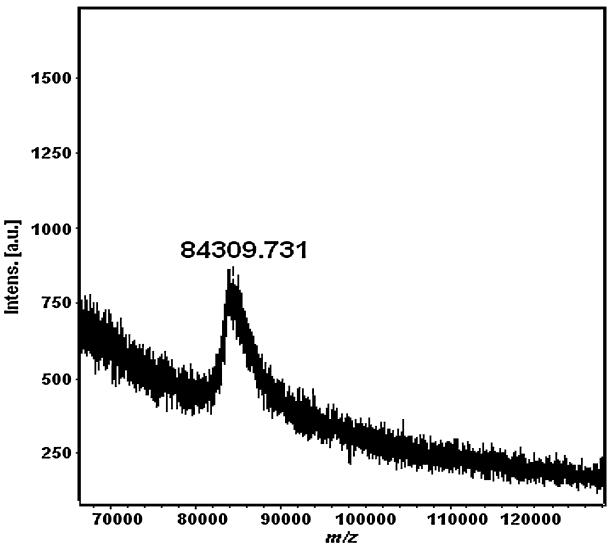

Figure 3 MALDI–TOF mass spectra of purified PPO from *Helicoverpa armigera*.

Table 1 Purification of prophenoloxidase (PO) from *Helicoverpa armigera*

Sample	Volume (ml)	Total protein (mg)	Total activity† (U/min)	Sp activity (U/min/mg)	Purification fold	Recovery (%)
Crude	42	160	63000	393	1	100
Ammonium salt ppt	9	28	49500	1767	4.4	78.5
Blue Sepharose CL-6B	4.5	2.5	40500	16200	41.2	64.2
Phenyl Sepharose CL-4B	2	0.9	31000	28181	71.7	49.2

†PO activity was determined by activation of PPO after each purification step with CPC (cetylpyridinium chloride).

A

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Ha PPO    YQRVSNAIGNRFGSDAGR-----------------------------------LIDIF
Bm ppo    YQRVSNAIGHRFGSNACRMIPIRNIALPNLDLPMELPYNEQFSLFVPKHRKLAGRLIDIF
Ha PPO    MGMR-------------------------------------------------FMDPKV
Bm PPO    MGMRDVEDLQSVCSYCQLRINPYMFNYCLSVAILHRPDTKGLSIPTFAESFPDKFMDPKV
Ha PPO    FR--------------MPIVIPSNYTASDTEPEQR-------------------------
Bm PPO    FRQAREVSSVVPSGARMPIVIPSNYTASDAEPEQRVAYFREDIGTNLHHWHWHLVYPFDA
Ha PPO    ------------------------YNVERMCNNL-------------------------
Bm PPO    ADRALVNKDRRGELFYYMHQQMIARFNIERFCNDLKKVETYSDFRGPIKEGYFPKMDSQV
Ha PPO    -------RFAGTTIRDLDRPVDQIR----------DRFLQAIENMSVMLPNG--------
Bm PPO    ASRAWPPRFAGTTIRDLDRPVDQIRSDVSELETWRDRFLQAIENMSVMLPNGRQLPLDEE
Ha PPO    ------------------------------------------RHLEQFGVMGDSATAMRDP
Bm PPO    TGIDVLGNLMESSIISRNRPYYGDLHNMGHVFISYSHDPDHRHLEQFGVMGDSATAMRDP
Ha PPO    VFY-----------------------RLDFPNI--------------------------
Bm PPO    VFYRWHAYIDDIFHLYKYKLTPYGNDRLDFPNIRVSSVSIEGGGTPNTLNTLWEQSTVDL
Ha PPO    -RGMDFTPRGSVLAR
Bm PPO    GRGMDFTPRGSVLAR
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B

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Hapropo ----------------------------------RHLEQFGVMGDSATAMRDPVFY------
Hvppo   ------ASILSPNRELYGSIHNNGHSFSAYIHDPTHRYLESFGVIADEATTMRDPFFFRWHAWI
Happo   ----LEASILSPNRELYGSIHNNGHSFSAYIHDPTHRYLESFGVIADEATTMRDPFFFRWHAWI
Bmpropo LGNLMESSIISRNRPYYGDLHNMGHVFISYSHDPDHRHLEQFGVMGDSATAMRDPVFYRWHAYI
Spdfr   LGNMMESSIISPNRGYYGDLHNMGHVFISYSHDPDHRHLEQFGVMGDSATAMRDPVFYRWHSYI
Ploint  LGNLMESSVISRNRAYYGDLHNMGHVFISYAHDPDHRHLEQYGVMGDSATAMRDPVFYRWHAYI
Pluxyl  LANLMESSIISRNRAYYGDLHNMGHVFISYAHDPDHRHLEQFGVMGDPATAMRDPIFYRWHAYV
Gmpropo LGNLMESSILSLNRGYYGDLHNMGHVFIAYSHDPDHRHLEEYGVMGDSATAMRDPVFYRWHAYI
Mspropo LGNLMESSIISPNRGYYGDLHNMGHVFAAYTHDPDHRHLEQFGVMGDSATAMRDPFFYRWHRFV
Mdpropo ------ASIISPNQSVYGDFHNMGHVFISYAHDPDHRHLESFGVMGDSATAMRDPVFYRW----
                                                 Copper binding B site
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Figure 4 Partial amino acid sequence alignment of PPO from *Helicoverpa armigera*. (A) Tryptic peptide sequences of PPO matched with the deduced sequences of phenoloxidase subunit I of *B. mori*. (B) Alignment of the putative conserved domains of the Copper Binding B site of *Helicoverpa armigera* prophenoloxidase with other insect species are underlined. Bmppo, *Bombyx mori* (GenBank No. AAG09304.1); Hvppo, *Heliothis virescens* (GenBank No. ABM65701.1); Happo, *Heliocverpa armigera* (GenBank No. ABU98653.1); Msppo, *Manduca sexta* (GenBank No. AAC37243.1); Gmppo, *Galleria mellonella* (GenBank No. AF336289_1); Mdppo, *Musca domestica* (GenBank No. AAR84669.1); Ploint, *Plodia interpunctella* (GenBank No. AAU29555.1) Pluxyl, *Plutella xylostella* (GenBank No. ADA60206.1) Spdfr, *Spodoptera frugiperda* (GenBank No. ABB92835.1)

to the hemocyanin M superfamily. The PPO of *H. armigera* showed homology with phenoloxidase subunit-I of *Bombyx mori*, putative conserved domains like copper-binding site (B) also matched with other arthropod PPOs (Fig. 4a,b, respectively).

Kinetic properties and substrate specificity

The effect of pH on *H. armigera* PPO activity was studied by using dopamine (2 mM) as a substrate at various pH (5.0 –10.0). The bell shaped pH activity profile of the purified PO is shown in Figure 5. Purified PPO is active in a pH range of 5–8, with maximum activity at pH 6.8 (Fig. 5). The enzyme showed typical Michaelis–Menten kinetics. The kinetic parameters calculated using 20 µl of enzyme for the substrate oxidation were Vmax 341U min^{-1} and Km 1.7 mM for dopamine, Vmax 229U min^{-1} and Km 10 mM for catechol (Table 2). The catalytic efficiency calculated from Vmax/Km showed the affinity of the enzyme for dopamine to be 5.8 folds higher than that of catechol.

Inhibition studies

Inhibition of PO activity was observed with various inhibitors such as phenylthiourea, O-phenanthroline, N-bromosuccinimide and sodium azide to an extent of 95, 95,

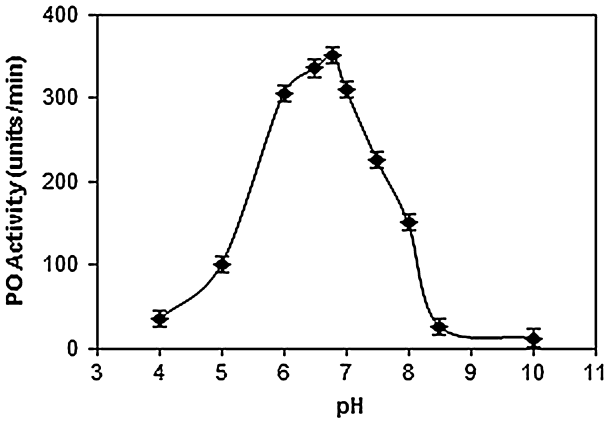

Figure 5 Effect of pH on phenoloxidase activity of *Helicoverpa armigera*. Buffers used were sodium acetate for pH (5.0–6.0), sodium phosphate for pH (6.0–7.0), Tris-HCl for pH (7.0–8.0), glycine-NaOH for pH (8.0–9.0). Error bars represent ±SD of three replications.

80 and 28%, respectively, at 1 mM concentration. Kojic acid, thiourea and dithiothreitol completely inhibited PO activity as shown in Table 2. Kojic acid was a competitive inhibitor, as increasing the kojic acid concentration (33 µM–330 µM) resulted in a family of lines with common intercepts on 1/V axis. The Ki value was found to be 47 µM (Fig. 6).

Table 2 Effect of various inhibitors on phenoloxidase activity of *Helicoverpa armigera*

Inhibitor (1 mM)	Inhibition rate* (%)
Control	0
Phenylthiourea (PTU)	95
Thiourea	100
Kojic acid	100
N-Bromosuccinimide (NBS)	80
O-Phenanthroline	95
Dithiothreitol (DTT)	100
Sodium azide	28

*The values are mean of three different assays.

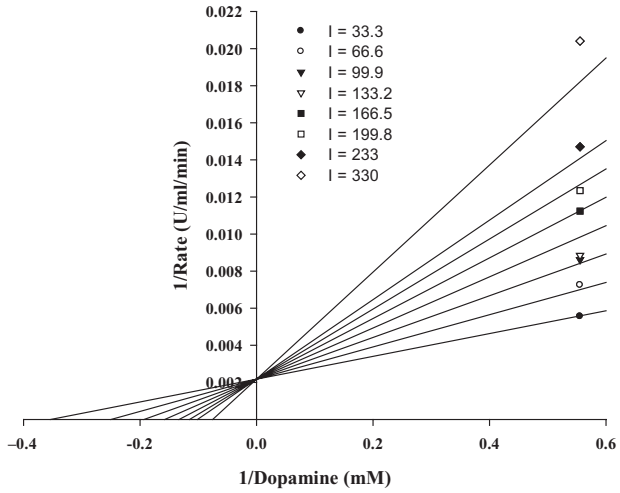

Figure 6 Lineweaver–Burk plot of PPO inhibited by Kojic acid. Concentrations of Kojic acid for curves 1–8 were 33.3, 66.6, 99.9, 133, 169, 199, 233 and 330 μM respectively. The inset represents the concentrations of Kojic acid in μM.

Discussion

Several studies have been conducted on various aspects of phenoloxidase from arthropods, but information on this enzyme in *H. armigera* is quite scanty. Therefore, there is a need to generate more information on the biochemical properties of *H. armigera* PO. Since the active PO enzyme is produced by proteolytic cleavage of a peptide from the proenzyme, one would naturally expect the molecular weight to be less than 80 kDa. In contrast, the approximate molecular weight of PO was found to be around 400 kDa, indicating self-polymerization with other hemolymph proteins (Beck *et al*. 1996). The proenzyme, once activated, cannot be purified because activated POs interact with other proteins forming protein complexes, and tend to stick on columns during chromatography. Therefore, the key to achieving PO purification is to minimize enzyme activation during the purification process. Hence, PO activating endogenous enzymes were removed by passing through a blue sepharose CL-6B column (Tong *et al*. 2005). Subsequently, PO free of the endogenous activating system was purified by a phenyl sepharose CL-4B column (Feng *et al*. 2008). The results showed that the protocol using blue sepharose CL-6B, followed by phenyl sepharose CL-4B was effective in purification of PO, as evidenced by high-specific activity and recovery of the enzyme.

Under reducing conditions (SDS-PAGE), purified PO showed a single polypeptide chain with a molecular weight of 85 kDa. Molecular weight of PO from *H. armigera* was also confirmed using MALDI–TOF MS and LC–ESI MS, and the molecular masses were 84 309 Da and 84 688 Da, respectively, which is in close agreement with those reported in other insect species (Ashida *et al*. 1990; Kopacek *et al*. 1995). The charge distribution in LC–ESI MS spectra confirmed the purity of the protein. The molecular mass of PPO from *H. armigera* was comparable with that of the other arthropod PPOs such as *Bombyx mori* (80 kDa) (Ashida 1971); *Calliphora vicinea* (87 kDa) (Naqvi & Karlson 1979); and *Maduca sexta* (77 & 71 kDa) (Hall *et al*. 1995). The PPO isolated from *H. armigera* in the present study seems to be a monomeric protein. Similarly, PPOs from *Heliothis virescens* (Lockey & Ourth 1992), *Hyalophora cecropia* (Andersson *et al*. 1989), *Blaberus discoidalis* (Durrant *et al*. 1993), *Locusta migratoria* (Cherqui *et al*. 1996) and *Neobellieria bullata* (Chase *et al*. 2000) are also monomeric proteins. The molecular mass of the PPO depends on isoforms, activation state and the animal species, which indicates the evolution or compatibility of the enzyme with regard to species physiology and associations with the environment (Asada & Sezaki 1999).

The purified PPO exhibited sequence homology with the phenoloxidase subunit I of *B. mori*. The deduced amino-acid sequence revealed that the conserved putative domains matched with the copper-binding B site. In comparison with *Heliocverpa armigera* and *Heliothis virescens* the present HaPPO is very similar to *Bombyx mori* and other arthropod PPOs. The overall sequence homology between *H. armigera* PPO and arthropod hemocyanins ranged from 29 to 39%.

The Km values of *H. armigera* PPO for dopamine and catechol were 1.7 and 10.0 mM, respectively. Our results show that the affinity of *H. armigera* PPO for dopamine is 5.8 folds higher than that of catechol, and these results corroborate with the studies of other insect species (Asada & Sezaki 1999; Feng *et al*. 2008). In general, L-DOPA has traditionally been used as a substrate for characterization of POs from arthropods (Brivio *et al*. 1996). The present study clearly indicates that the PPO from *H. armigera* does not utilize L-DOPA as a substrate. The *H. armigera* PPO was inhibited by phenylthiourea, thiourea and Kojic acid. Similar inhibition has also been reported for *Heliothis virescens* PPO

(Popham *et al*. 2004). Kojic acid is a competitive inhibitor of PPO. The other compounds are known for their chelating properties in copper-containing metalloproteins (Li & Kubo 2004). N-bromosuccinimide (NBS) and O-phenanthroline also inhibit PPO activity. Inhibition by NBS is due to the modification of tryptophan residues (Pang *et al*. 2005). O-phenanthroline is a chelator of iron cations, suggesting that a few iron atoms may exist in PPO. Dithiothretal completely inhibited the PPO, whereas sodium azide inhibited poorly at 1 mM concentration. Similar results were observed from *Amphioxus* spp. (Pang *et al*. 2005). The PPO is considered to be an integral part of the insect host defense system. This is the first report where PO from *H. armigera* was purified and characterized. Results obtained in this study provide a basis for understanding the role of PPOs in physiology and development of *H. armigera*.

Acknowledgments

We are thankful to University Grant Commission (UGC) for providing the fellowship under UGC-RFSMS scheme during this work. This research was supported by a research grant from the Department of Science and Technology (DST), New Delhi, to K. Sreeramulu and University Grant Commission under Special Assistance Program (UGC-SAP), New Delhi, India. We also thank the Director Rabindra R. J and Dr. Jalali, S. K., Principal Scientist, NBAII, Bangalore, India, for providing the lab facilities throughout this work.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. LC–ESI mass spectrum of purified PPO sample. Inset: deconvoluted mass of 84688.3 Da