

Immune cascade of *Spodoptera litura*: Cloning, expression, and characterization of inducible prophenol oxidase

R. Rajagopal, K. Thamilarasi, G. Raja Venkatesh, P. Srinivas, Raj K. Bhatnagar *

International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi 110 067, India

Received 27 August 2005

Available online 19 September 2005

Abstract

Haemolymph associated phenol oxidase is a critical component of invertebrate immune reaction and cuticle sclerotization. Phenol oxidase catalyses the conversion of mono-phenols to diphenols and quinones which finally leads to melanin formation. We have cloned the c-DNA encoding phenol oxidase from the haemocytes of *Spodoptera litura* and expressed it in *Escherichia coli*. The encoding gene is 2452 bp with an open reading frame of 2091 bp translating into a 697 amino acid protein. Multiple alignment analysis of the predicted protein sequence shows close homology to other lepidopeteran PPOII type genes. The transcription of the gene is induced upon microbial challenge of 6th instar larvae with *E. coli* and is unresponsive to injury. Cloning of the ORF of *SLPPO* in-frame in the *E. coli* expression vector pQE30 resulted in its expression. Enzymatic analysis of the recombinant protein reveals that the recombinant protein is catalytically active on 4-methyl pyrocatechol upon activation by cetyl pyridinium chloride.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Insect immunity; Prophenol oxidase; Melanization; *Spodoptera litura*; Haemocytes

Insects are the most abundant and diversified group of organisms, which have acclimatized and colonized practically all ecological niches. They have a robust immune system permitting them to occupy diverse habitats. Insect's immune response is primarily innate consisting of humoral and cellular factors. Besides structural barriers, the various mechanisms by which the insects protect themselves against microbial challenge include among others antibacterial peptides and proteins like cecropins, attacins, defensins, etc., and cellular immune responses like phagocytosis, melanotic encapsulation, coagulation, and nodule formation [1].

A typical response of insects to invading microorganisms is encapsulation wherein cellular layer of haemocytes is deposited around the microbe and is subsequently rendered ineffective by melanization [2,3]. Melanin deposition is initiated by the action of phenol oxidase on mono-phenols and o-diphenols which are converted to quinones and finally into melanin. The central enzyme in melanin biosynthesis

pathway is phenol oxidase [4], which is normally present in the haemolymph as an inactive precursor, and is activated by proteases in a specific and regulated process [5]. It has been reported for several insects that the level of phenol oxidase activity increases following challenge of insects with bacteria, parasites or bacterial cell wall components [1]. The initial recognizing molecules activate the PPO-activating cascade, resulting in the rapid increase in transcripts of the prophenol oxidase activating enzyme (PPAE) [6]. The increased expression of PPAE leads to activation of PPO resulting in higher phenol oxidase activity.

Considering the central role of phenol oxidase in the immune response of insects, a lot of studies have been conducted towards its molecular characterization and regimen of its induction. Various components of the immune cascade pathway have been identified and their temporal regulation studied. Accordingly, two isozymes of prophenol oxidases PPO I and PPO II have been isolated from a number of different insects including *Manduca sexta*, *Bombyx mori*, *Sarcophaga bullata*, *Lymantria dispar*, *Hyphantria cunea*, and many species of *Anopheles*.

* Corresponding author. Fax: +91 11 616 2316.

E-mail address: raj@icgeb.res.in (R.K. Bhatnagar).

To understand the immune mechanism in the insect *Spodoptera litura*, one of the most important polyphagous agricultural pests of the developing world, we have cloned the cDNA of a prophenol oxidase. We have studied the activation of this gene at the transcript level following microbial challenge of the insect. Further, we have expressed it in a catalytically active state in a bacterial expression vector. One of the major limitations of searching for inhibitors against most of the critical proteins of insect immune response has been non-availability of phenol oxidase in a catalytically active state. Thus, for the first time we have been able to express phenol oxidase which could be catalytically activated in vitro.

Materials and methods

Insect rearing. *Spodoptera litura* larvae were reared from eggs in our laboratory on fresh Castor leaves (*Ricinus communis*) under a photoperiod of 14:10 (light:dark), 70% relative humidity, and 27 °C. Sixth instar 1st day larvae were used in our experiments.

Haemolymph collection and haemocyte separation. *S. litura* larvae (sixth instar, second day) were pierced in their prolegs with a sterile needle and the haemolymph collected into pre-chilled anticoagulant buffer. The haemolymph was resuspended in the anticoagulant buffer and spun at 700g for 5 min at 4 °C. The supernatant was discarded and the pellet containing the haemocytes was resuspended in Trizol reagent and processed for RNA extraction, according to the manufacturers protocol (Invitrogen, USA). Alternatively, the haemocytes were snap-frozen in liquid nitrogen and stored at –70 °C until further use.

Cloning of *S. litura* prophenol oxidase. The cDNA was synthesized using a poly(dT) primer with 4 µg of total RNA and reverse transcriptase (Superscript II, Invitrogen) according to the manufacturer's instruction. Clustal alignment of prophenol oxidases of different insects revealed regions of extensive homology. Degenerate oligonucleotide primers were designed from two conserved regions in insect prophenol oxidases coding for the following conserved amino acids HHWHWH and DPVFTR. Polymerase chain reaction was performed using degenerate primers and cDNA prepared from total RNA of haemocytes as template. A 600 bp fragment was amplified and cloned into pGEMT-easy vector (Promega) and sequenced by the dideoxy chain termination method of Sanger et al. Sequence similarity searches were carried out using the program BLAST (www.ncbi.nlm.gov/BLAST/). On the basis of the sequence of the cloned insert, gene specific primers were designed in the reverse direction for 5' RACE (rapid amplification of cDNA ends) and in the forward direction for 3' RACE. The 3' and 5' ends of the gene were amplified following the protocol described in RACE kit manuals (Invitrogen). The PCR-amplified fragments were cloned into pGEMT easy vector and sequenced as described before.

On the basis of the sequence of 5' and 3' RACE products, gene specific end primers corresponding to the 5' and 3' end of the ORF were synthesized. These primers were used in a PCR using single stranded cDNA as template to yield a 2.1 kb fragment of the target gene (*S. litura* prophenol oxidase II). This PCR product was cloned into pGEMT-easy vector and sequenced by primer walking method. Ten independent clones of the gene *slppo* were sequenced to yield identical sequencing thus confirming the fidelity of PCRs.

Expression of *S. litura* PPO in *Escherichia coli*. *S. litura* prophenol oxidase gene (PPO) was cloned in pQE30 with a N terminal 6x His-tag as a *Bam*HI and *Kpn*I fragment. *E. coli* M15 cells were transformed with this recombinant plasmid. Freshly inoculated cultures were grown until A_{600} reached an OD of 0.5, following which it was induced with 1 mM IPTG and allowed to grow further for 3 h. The cells were pelleted, resuspended in 50 mM sodium phosphate buffer (pH 7.5) containing 8 M urea, and mixed completely in a nutator at 28 °C (room temperature-RT) for 1 h. This was then centrifuged at 12,000 rpm for 15 min at RT and the supernatant fraction contained the solubilized protein was recovered. The

protein was purified by binding the supernatant to Nickel-NTA in a nutator for 1 h. The slurry was loaded on a Bio-Rad column and extensively washed with buffer (50 mM sodium phosphate, pH 7.5). The protein was eluted in buffer containing 250 mM imidazole and the salt was removed by dialysing with 50 mM sodium phosphate, pH 7.5, with three changes.

Phenol oxidase assay. To examine whether the *E. coli* expressed protein was catalytically active, it was subjected to a phenol oxidase assay as described in [7], with minor modifications. 1 ml of reaction mixture containing 0.5, 1, 1.5, and 2 µg protein with 0.02% CPC (cetylperidinium chloride), in buffer incubated at RT for 10 min and finally 2 mM substrate (4-methyl pyrocatechol) was added and the increase in OD at 528 nm was read. A blank without protein served as a control. PPO inhibition assay was performed using the specific inhibitor phenyl thio-urea (PTU) at 5 mM concentration in the reaction mixture containing 1 µg of the enzyme protein. All the experiments were carried out at RT. Specific activity of PPO was calculated using extinction coefficient of the substrate at 528 nm as $4230 \text{ M}^{-1} \text{ cm}^{-1}$ [8].

Northern blot analysis. *S. litura* 6th instar, 1st day larvae were injected with 1×10^6 *E. coli* K-12 cells suspended in Ringer's solution, using a microinjector (KPS 210, KD scientific, Newhope, PA, USA). Mock injury on insects was done by injecting Ringer's solution alone. Haemolymph was collected from the experimental insects at 2, 4, 10, 12, and 24 h post-infection and haemocyte was separated. Total RNA was extracted from the haemocyte as mentioned earlier. Equal amounts of RNA for each treatment were separated by denaturing electrophoresis in 1% agarose-formaldehyde gel with Mops buffer and then transferred to nylon membrane. The blot was stained with methylene blue to quantify the amount of RNA loaded in each treatment. After washing of the methylene blue, the blot was then hybridized with random-primed ^{32}P *slppo1* gene in pre-hybridization solution. The blot was then washed with $1 \times \text{SSC}$ and 0.1% SDS and exposed to auto-radiographic film at –70 °C for 5 days.

Real time analysis. *E. coli* K-12 cells were subcultured from overnight stock and were grown for 4 h. These cells were pelleted and resuspended in sterile Ringer's solution to a concentration of 2×10^9 cells/ml. Sixth instar second day larvae were injected with this inoculum (5 µl/insect) by piercing through the proleg using a microinjector (KPS 210, KD scientific, Newhope, PA, USA) with Hamilton syringe. Five microliter plain Ringer's solution was injected in saline control insects. Haemolymph from different stages of insect larvae, saline injected, and *E. coli* injected larvae after 6 and 18 h was collected in pre-chilled anticoagulant buffer and spun at 700g for 5 min at 4 °C. The haemocytes were stored at –70 °C, if not used immediately. The haemocyte pellet was homogenized in Trizol and RNA was isolated according to the manufacturer's instructions (Invitrogen, USA). Contaminating genomic DNA was removed by treating with RQ1 DNase (Promega, USA) at 37 °C.

Real time analysis was done using Quantitect SYBR Green RT-PCR kit (Qiagen, Germany) in the iCycler iQ system (Bio-Rad). The sequences of the primer pair used for amplifying β -actin gene are forward: 5'-CAG ATC ATG TTT GAG ACC TTC AAC -3' and reverse: 5'-GA/C/TC CAT CTC C/TTG CTC GAA A/GTC-3'. The primer pair for amplifying PPO gene is forward: 5'-AAC CAA CTG AGT TGC GTT GA-3' and reverse: 5'-AAC TGC TGA TGG TGG GTT TCT A-3'. Each 20 µl reaction contained 2 µl RNA template (100–500 ng), 10 µl of $2 \times$ QuantiTect SYBR Green RT-PCR Master Mix, 10 picomoles of each appropriate primer, 0.2 µl of QuantiTect RT Enzyme Mix, and DEPC treated water to make up the volume. Real time cycler conditions used were as follows: preliminary reverse transcription at 48 °C for 30 min, an initial activation step at 95 °C for 15 min and 40 cycles of denaturation at 95 °C, annealing at 52 °C, and extension at 72 °C for 30 s each followed by gradual temperature increase from 50 °C to 94 °C at the rate of 1 °C/10 s to enable melt-curve data acquisition. A non-template control was run with every real time PCR experiment. Each experiment was carried out in duplicate. The threshold cycles (C_T) were obtained for PPO and β -actin transcripts from each experiment. Difference between C_T of the reference gene, β -actin, and the gene of interest, PPO (ΔC_T) was determined and the relative abundance of the transcript was calculated following comparative C_T method using the formula $2^{-\Delta C_T}$ [9].

Results

Molecular cloning of *slppo* cDNA

Based on the CLUSTAL alignment of reported sequences of prophenol oxidase genes from different insect orders, degenerate primers were designed to identify the conserved region of prophenol oxidase encoding gene. Amplification using degenerate primers and haemocyte cDNA as template yielded a 600 bp fragment, which was cloned in pGEMT-easy and sequenced. Homology search of the sequence through NCBI BLAST revealed similarity with other insect prophenol oxidase. Specific primers were designed, and the 5' and 3' ends of the gene were amplified by following the RACE protocol.

The deduced amino acid sequence in the ORF (Fig. 1) of the gene starts from the 82nd nucleotide and is 2091 bp long encoding a 697 amino acid long protein. The 3'

untranslated region extends from 2174th nucleotide and is 239 nucleotides long containing a putative polyadenylation sequence. The calculated molecular weight of the deduced protein is 79,865 Da. The gene sequence has been deposited in the genBank and has the Accession No. [AY703825](#).

Comparison of the amino acid sequence of *slppo* with that of other insect PPO

Using Clustal W alignment program (MacVector, version 7), the deduced amino acid sequence of *S. litura* PPO I (*slppo*I) was aligned with that of other insect prophenol oxidase. The alignment was carried out with the following parameters. Similarity matrix-blosum, open gap penalty of 10.0 and extended gap penalty of 0.1. The pairwise alignment showed that SLPPO1 exhibits 80% homology to PPO II of *B. mori* and *M. sexta*, while it shows only



Fig. 1. Nucleotide and deduced amino acid sequence of *S. litura* prophenol oxidase cDNA. Amino acids are represented by one letter abbreviations. Copper binding sites I and II are underlined and the potential cleavage site for the activation to PO is boxed and shown with arrow. The potential polyadenylation signal (AATAAA) is indicated in bold in the 3' region.

40% similarity with PPO I. Analysis of the protein sequence shows that there is no signal sequence at the N terminus, while it predicted a proteolytic cleavage site between amino acid position 55 and 56 represented by arginine (R) and phenylalanine (F), respectively (Fig. 2). The predicted protein also contains the two conserved copper binding

motifs characteristic of arthropod phenol oxidase. The N terminal sequence of SLPPO1 is different from that of other reported insect PPO by having a sequence MSDMSGDVVEH (from amino acid 1 to 11). The initial amino acid sequence of MSD is a characteristic of PPOI type of prophenol oxidase. While SLPPO1 shows this

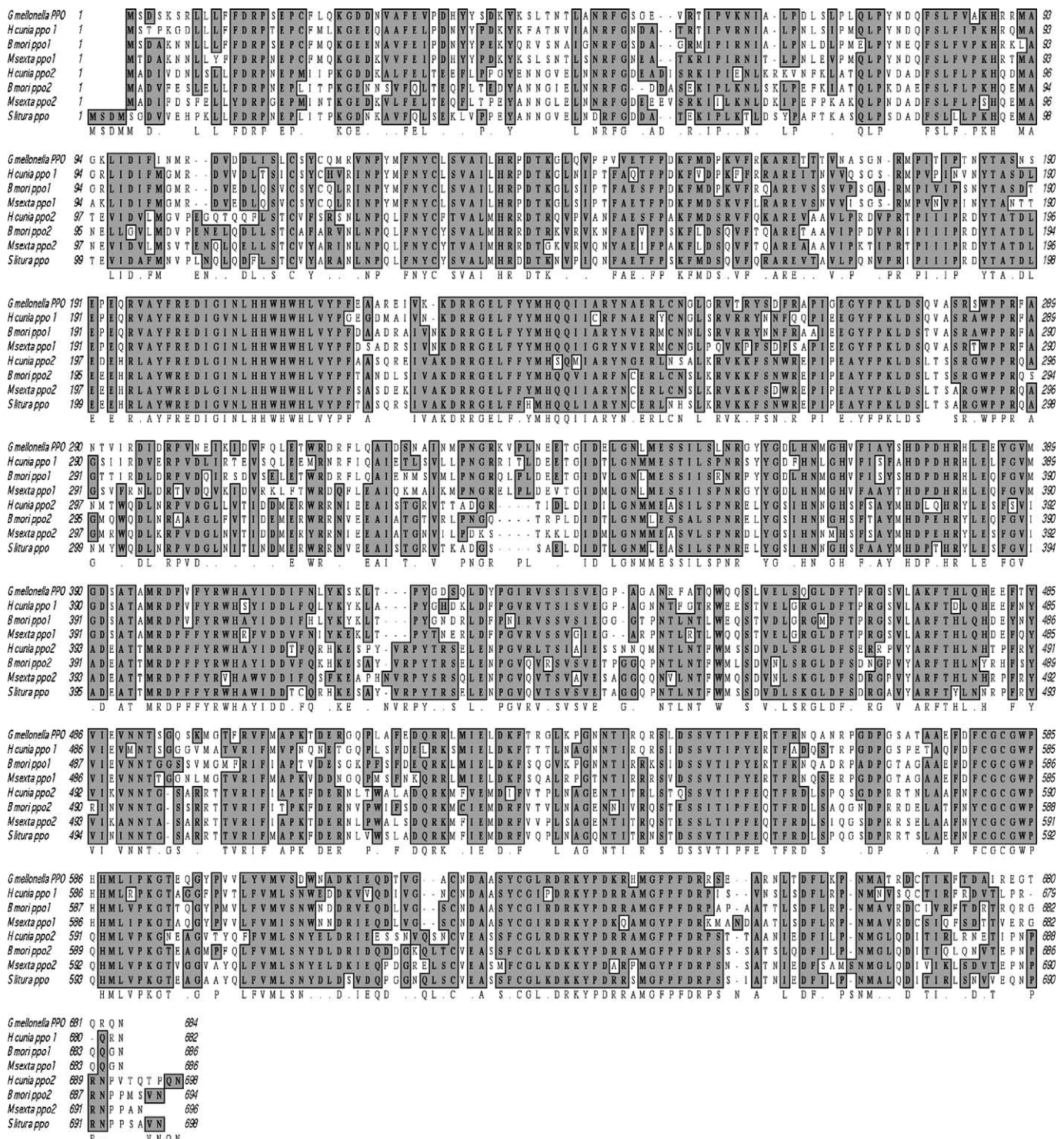


Fig. 2. Comparison of published lepidopteran prophenol oxidase amino acid sequences. The sequences compared are *B. mori* PPO I and PPO II, *M. sexta* PPO I and II, *H. cunea* PPO I and II, *G. mellonella* PPO, and *S. litura* PPO. Highly conserved copper binding motifs are underlined.

characteristic, the rest of its sequence aligns with PPO II type phenol oxidase from insects (Fig. 2).

Presence of *slppo1* gene in different growth stages

Haemolymph could be collected only from the fourth larval instar of the insect. QRT-PCR of the haemocytes for the *slppo1* gene transcript, on all days from the fourth through the sixth instar of *S. litura* shows that it is highly abundant on the fifth instar. While the transcript levels reduce on the 6th instar 1st day, it becomes significant on the next day while on the following two days it tapers off (Fig. 3). Thus, we find that the level of *slppo1* varies at different larval stages and is maximum during the active feeding period.

Induction of *slppo1* upon microbial challenge of *S. litura*

Spodoptera litura 6th instar 1st day larvae were challenged with actively growing *E. coli* K-12 and analysed for the induction of *slppo1*. A positive control of challenging insects only with Ringer's solution and a negative control of no challenge/injury showed almost equal amount of *slppo1* transcript, while *E. coli* challenged insects showed a significant increase in the *slppo1* transcript level. The transcript level increased within 2 h of bacterial challenge and remained at the elevated level upto 16 and 24 h post-challenge (Fig. 4A). This establishes that *slppo1* gene is induced immediately upon microbial challenge. The Real Time PCR data also supported the inducibility of *slppo1* transcript. The abundance of the *slppo1* transcript was compared in bacteria injected and saline injected larvae with naïve larvae after the amount of RNA in all the three samples was normalized with internal β -actin control. Six hours after injection the *slppo1* transcript increased up to 2.6-fold as compared to saline control and after 18 h it increased up to 10.19-fold (Fig. 4B).

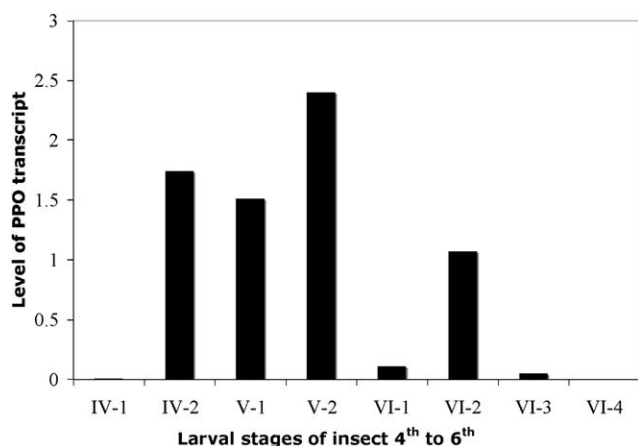


Fig. 3. Transcript abundance of *slppo1* in different larval stages of *S. litura*. Haemocytes were collected from each day from 4th instar until the last day of 6th instar and total RNA extracted. The levels of *slppo1* transcript in different insect larvae were analysed by real time PCR using the SYBR green fluorescent probe.

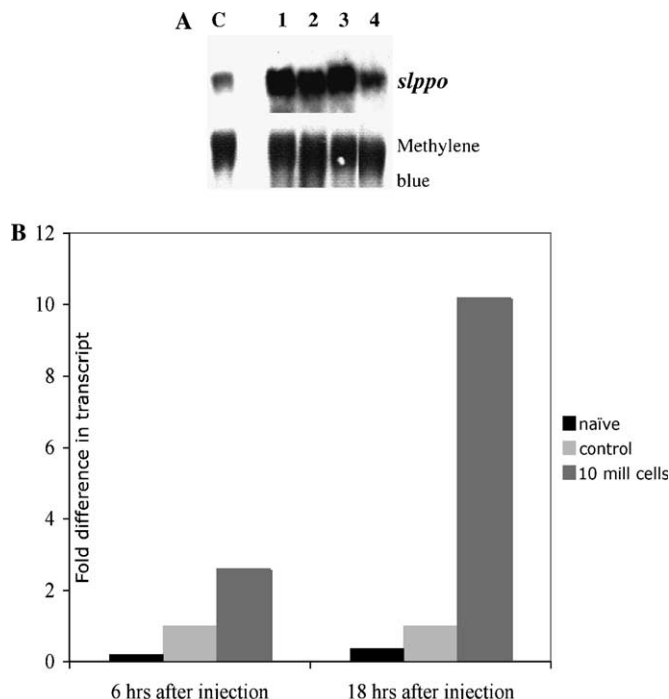


Fig. 4. Induction of *S. litura* PPO gene following microbial challenge. (A) Northern blot analysis of total RNA extracted from control haemocytes C, following infection with *E. coli* after 2 h (1), 16 h (2), and 24 h (3) and following mock infection with Ringer's solution alone (4). Fifty microgram of RNA was loaded in each lane, separated and transferred to nylon membrane, and probed with a P^{32} labelled *S. litura* PPO cDNA. The blot was stained with methylene blue (0.04%) before probe hybridization and shows an equal amount of total RNA loaded in each well. (B) Real time quantitative analysis of *slppo1* transcript after infection with *E. coli* at different time periods.

Expression of *slppo1* in *E. coli* and its enzymatic activity

The *slppo1* was cloned in-frame into pQE 30 vector. Induction of the recombinant clone with IPTG resulted in strong accumulation of the protein corresponding to the calculated molecular mass of 79 kDa. Fractionation of cells revealed its presence in the inclusion bodies only. Inclusion bodies were solubilized in 8 M urea and purified to near homogeneity. Western analysis with anti-PPO antibodies (raised against *M. sexta* PPO) revealed the presence of protein at expected size (Fig. 5).

To evaluate whether the purified protein was in a catalytically activatable state, it was activated by the routinely employed PPO activation detergent, CPC. Activation of the expressed protein with 0.02% CPC and its incubation with 2 mM 4-methyl pyrocatechol in 50 mM sodium phosphate buffer resulted in an increase in absorbance at 528 nm. The absorbance increased linearly with time and protein concentration. The negative control of not activating the protein with CPC and without protein did not result in significant increase in absorbance. The expressed enzyme was catalytically active and metabolized 4-methyl pyrocatechol (specific activity 296 $\mu\text{m}/\text{min}/\text{mg}$ protein). The hydrolysis of 4-methyl pyrocatechol was completely inhibited by the phenol oxidase inhibitor phenyl thio-urea (PTU).

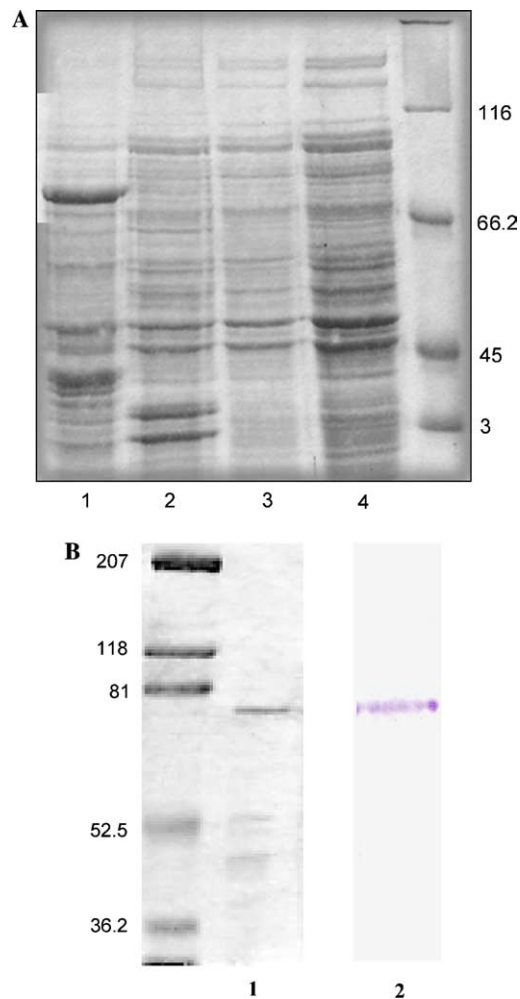


Fig. 5. Heterologous expression and purification of SLPPO. (A) *slppl1* was cloned in *E. coli* expression vector pQE 30 and was induced with IPTG and resolved into inclusion bodies (lane 1) and supernatant fractions (lane 2). Similarly, the vector alone without any insert was grown and processed into inclusion bodies (lane 3) and supernatant (lane 4). (B) The IB were solubilized and the protein partially purified. Lane 1, Coomassie staining of the purified protein; lane 2, the protein detected with anti-PPO antibodies.

Discussion

Melanotic encapsulation is one of the most important defence responses by invertebrate organisms to protect themselves from microbial challenge, and the key enzyme involved in melanization is prophenol oxidase [10]. It is a metalloenzyme (monophenol, L-dopa: oxidoreductase; EC1.14.18.1) that catalyses the hydroxylation of tyrosine to dopa and the oxidation of phenols to quinones [11]. Insect PPO are proenzymes, are devoid of a N terminal signal sequence, and have two copper binding sites in the active site of the enzyme. There is an increase in the transcript level of this enzyme following parasitic invasion and insects having impaired machinery to produce this enzyme show reduced ability to suppress pathogen attack by melanizing and encapsulating them [12].

Lepidoptera is an order of insect, which contains some of the most important and successful animals, and the underlying basis is speculated to be a robust immune system. Prophenol oxidase cDNA has been isolated and analysed from *Galleria mellonella* [13], *B. mori* [14], *M. sexta* [7,15], and *H. cunea* [16] from the order Lepidoptera. We have cloned the cDNA for prophenol oxidase from the common cutworm *S. litura* which is one of the most important polyphagous agricultural pests of the developing world. The cDNA is 2400 bp long and encodes an ORF from nucleotide position 83 to 2174 which gets translated into a 697 amino acid long protein. The deduced amino acid sequence has characteristic of insect prophenol oxidases in having two copper binding motifs with a calculated molecular mass of 79 kDa. The deduced protein sequence of SLPPO is very similar to that of other lepidopteran PPO II, from *B. mori*, *H. cunea*, and *M. sexta*. The SLPPO is unique among lepidopteran insects in containing the characteristic amino acid sequence of PPO I in the N terminus, while the rest of the protein displays homology to lepidopteran PPO II.

When the insect recognizes a microbial challenge, the cascade of immune events begins immediately leading to simultaneous secretion of antimicrobial peptides as well as cellular responses like phagocytosis, encapsulation, and melanization. Upregulation of PPAE (prophenol oxidase activating enzyme) gene is one of the responses of immune challenge. This enzyme cleaves the pro-region of the PPO, which is already present in the haemolymph as an inactive zymogen, leading to its activation. Our results on analysing the transcript profile of SLPPO following inoculation with *E. coli* K-12 reveal that the gene is upregulated within 2 h of infection. Such an increase in PPO transcript is not encountered when *S. litura* larvae are injected with the buffer alone. Hence, the increase in PPO transcript is a result of microbial presence only. Interestingly, none of the earlier reports of Lepidopteran PPO indicate that the PPO gene can be induced after microbial infection. Conversely, in *M. sexta* there is an increase in the transcript levels of prophenol oxidase—activating proteinase (PAP) following microbial challenge [17]. It is hypothesized that PPO in the haemolymph is released by cell burst from the cytoplasm of the haemocytes and exists in the haemolymph as an inactive zymogen. It is converted into its active form by cleavage of its pro-region by specific PAP, which in turn are regulated by other factors in the PPO-activating cascade. Also, anti-PPO II antibodies of *M. sexta* locate the PPO production cells as oenocytoids [15] while in *Galleria* they get located in granular cells [13]. Other reports have also suggested the increase in PPO transcripts in mosquito species following blood meal or infection [18]. Further, silencing of the isoform of haemolymph PPO in *Armigeres sublabatus* almost completely inhibited the melanization of *Dirofilaria immitis* microfilariae [12]. Thus, the role of PPO in insect's immune response and development is vital.

We have been able to express this enzyme in the *E. coli* expression vector pQE30. The recombinant protein is catalytically activatable with the detergent CPC. Consistent with the earlier reports from native PPO purified from insect haemolymph, the recombinantly expressed PPO catalysed 4-methyl pyrocatechol [7]. This is the first report of the expression of an insect PPO in an *E. coli* expression system, which is catalytically activatable. Availability of recombinantly expressed PPO in *E. coli* offers opportunity to understand the activation mechanism of a molecule critical for insect immunity. It also offers a system to screen for native and alien molecules, which interfere with the catalytic potential of PPO and lead to compromise in insect immunity.

Acknowledgment

We sincerely thank Dr. M.R. Kanost, Kansas State University, for providing the antibodies for PPO.

References

- [1] M. Ahida, P.T. Brey, Recent advances in research on the insect prophenol oxidase cascade, in: P.T. Brey, D. Hultmark (Eds.), *Molecular Mechanisms of Immune Responses in Insects*, Chapman & Hall, London, 1998, pp. 135–172.
- [2] J.P.N. Pathak, Cell mediated defense reactions in insects, in: J.P.N. Pathak (Ed.), *Insect Immunity*, Oxford and IBH Publishing, New Delhi, 1993, pp. 47–58.
- [3] M.D. Lavine, M.R. Strand, Insect haemocytes and their role in immunity, *Insect Biochem. Mol. Biol.* 32 (2002) 1295–1309.
- [4] M. Ashida, H.I. Yamazaki, Biochemistry of the phenoloxidase system in insects: with special reference to its activation, in: E. Onishi, H. Ishizaki (Eds.), *Moulting and Metamorphosis*, Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin, 1990, pp. 239–265.
- [5] K. Soderhall, L. Cerenius, Role of prophenoloxidase activating system in invertebrate immunity, *Curr. Opin. Immunol.* 10 (1998) 23–28.
- [6] Y. Wang, H. Jiang, M.R. Kanost, Expression and purification of *Manduca sexta* prophenoloxidase activating proteinase precursor (proPAP) from baculovirus-infected insect cells, *Protein Expr. Purif.* 23 (2001) 328–337.
- [7] M. Hall, T. Scott, M. Sugumaran, K. Soderhall, J.H. Law, Proenzyme of *Manduca sexta* phenol oxidase: purification, activation substrate specificity of the active enzyme and molecular cloning, *Proc. Natl. Acad. Sci. USA* 92 (1995) 7764–7768.
- [8] J. Nagai, S. Kawabata, A link between blood coagulation and prophenol oxidase activation in arthropod host defense, *J. Biol. Chem.* 275 (2000) 29264–29267.
- [9] M.W. Pfall, A new mathematical model for relative quantification in real time RT-PCR, *Nucleic Acids Res.* 29 (2001) 2002–2007.
- [10] M. Sugumaran, M.R. Kanost, Regulation of insect hemolymph phenol oxidases, in: N.E. Beckage, S.N. Thompson, B.A. Fredric (Eds.), *Parasites and Pathogens*, vol. 1, Academic Press, San Diego, 1993, pp. 317–342.
- [11] H.S. Mason, Oxidases, *Annu. Rev. Biochem.* 34 (1965) 595–634.
- [12] S.H. Shiao, S. Higgs, Z. Adelman, B.M. Christiansen, S.H. Liu, C.C. Chen, Effect of prophenol oxidase expression knockout on the melanisation of microfilariæ in the mosquito *Armigeres sublabatus*, *Insect Mol. Biol.* 10 (2001) 315–321.
- [13] D. Li, C. Scherfer, A.M. Korayem, Z. Zhao, O. Schmidt, U. Theopold, Insect hemolymph clotting: evidence for interaction between the coagulation system and prophenoloxidase activating cascade, *Insect Biochem. Mol. Biol.* 32 (2002) 919–928.
- [14] T. Kawabata, Y. Yasuhara, M. Ochiai, S. Matsura, M. Ashida, Molecular cloning of insect prophenol oxidase: a copper containing protein homologous to arthropoid hemocyanin, *Proc. Natl. Acad. Sci. USA* 92 (1995) 7774–7778.
- [15] H. Jiang, Y. Wang, C. Ma, M.R. Kanost, Subunit composition of prophenol oxidase from *Manduca sexta*: molecular cloning of subunit ProPO P1, *Insect Biochem. Mol. Biol.* 27 (1997) 835–850.
- [16] D.S. Park, S.W. Shin, M.G. Kim, S.S. Park, W.J. Lee, P.T. Brey, H.X. Park, Isolation and characterization of the cDNA encoding the prophenoloxidase of the fall wetworm *Hyphantria cunea*, *Insect Biochem. Mol. Biol.* 27 (1997) 983–992.
- [17] H. Jiang, Y. Wang, X.Q. Yu, M.R. Kanost, Prophenol oxidase activating proteinase 2 from haemolymph of *Manduca sexta*: a bacteria inducible serine protease containing two clip domains, *J. Biol. Chem.* 278 (2003) 3552–3561.
- [18] L.H. Huang, B.M. Christensen, C.C. Chen, Molecular cloning of a second prophenol oxidase cDNA from mosquito *Armigeres sublabatus*: prophenol oxidase expression in blood fed and microfilariæ-inoculated mosquitoes, *Insect Mol. Biol.* 10 (2001) 87–95.