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Armigeres subalbatus prophenoloxidase III: Cloning, characterization and potential role in morphogenesis

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ABSTRACT

It has long been suggested that phenoloxidases (POs) play key roles in various physiological functions in insects, e.g., cuticular sclerotization, wound healing, egg tanning and melanotic encapsulation of pathogens. Here we report that a mosquito PO, designated *Armigeres subalbatus* prophenoloxidase III (*As-pro-PO III*), is likely involved in the morphogenesis in mosquito. Expression profile analysis found that *As-pro-PO III* mRNA is persistently expressed in adult mosquitoes and is not significantly affected by blood feeding, microfilariae inoculation, or *Escherichia coli* inoculation, but expression levels of *As-pro-PO III* fluctuated in larval and pupal stages. Knockdown of *As-pro-PO III* expression in pupae using double-stranded RNA resulted in high pupal mortality and deformed adults that subsequently died following emergence. Promoter activity analyses by electrophoretic mobility-shift assays and transfection assays suggest that the *As-pro-PO III* gene is positively regulated by a putative Zeste motif, a developmental regulatory element. These results suggest that As-pro-PO III is associated with morphogenesis of mosquitoes.

1. Introduction

Phenoloxidase (monophenol, L-Dopa: oxidoreductase; EC 1.14.18.1; PO) is a multifunctional copper containing oxidase that catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (dopa) and the oxidation of ortho-diphenolic substances to their respective quinones that then polymerize to form melanin (Ashida and Yamazaki, 1990). In mosquito, POs are synthesized as inactive enzymes call prophenoloxidases (pro-POs), and activation involves limited proteolysis by a serine protease with trypsin-type activity (Ashida, 1990). Mosquitoes have more POs than other insects. For example, the genomes of *Anopheles gambiae* and *Aedes aegypti* contain nine and ten pro-PO genes, respectively (Christophides et al., 2002; Waterhouse et al., 2007). In *Armigeres subalbatus*, at least five distinct pro-POs have been identified, designated *As-pro-PO I–V* (Cho et al., 1998; Huang et al., 2001; Tsao et al., unpublished data). However, the genomes of *Drosophila melanogaster* and *Manduca sexta* have only three PO genes, respectively (Asada et al., 2003; Kanost et al., 2004). The importance of PO polymorphisms in mosquitoes is still unknown (Christensen et al., 2005), POs have been suggested to be involved in various biochemical processes for the successful survival of insects, including cuticular sclerotization, egg tanning, wound healing and melanotic encapsulation of parasites (see reviews by Ashida and Yamazaki, 1990; Christensen et al., 2005). The specific physiological function for an individual mosquito proPO *in vivo*, has rarely been clearly established (Kim et al., 2005). POs might either have specific biological functions or they might be multifunctional, with one function being primary (Christensen et al., 2005). Shiao et al. (2001) and Tamang et al. (2004) were able to use gene knockdown strategies *in vivo* to verify that *Ar. subalbatus* As-pro-PO I is involved in melanization immune responses against filarial worms. They demonstrated that *As-pro-PO I* transcripts significantly increase in abundance during melanotic encapsulation initiated by the intrathoracic inoculation of microfilariae, and that melanotic encapsulation of parasites was almost completely abolished in *As-pro-PO I* knockdown mosquitoes. Using biochemical purification and mass spectroscopy, Kim et al. (2005) also were able to identify a specific PO (*Ae. aegypti* prophenoloxidase 5, AePPO5) involved in egg chorion hardening in *Ae. aegypti*.

Although it has long been suggested that the functions of cuticular POs are for wound healing, defense, and cuticle sclerotization (Lai-Fook, 1966; Brunet, 1980; Ashida and Yamazaki, 1990; Ashida and Brey, 1995; Andersen et al., 1996; Marmaras et al., 1996; Lai et al., 2001), the relative roles of various POs might play in cuticular sclerotization in insect are still debatable. Recently, Arakane et al. (2005) demonstrated that another phenol-oxidizing

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enzyme Laccase 2, play a critical role in sclerotization of the red flour beetle, *Tribolium castaneum*. The data presented here, demonstrate that *Ar. subalbatus* prophenooxidase III (As-pro-PO III), is likely involved in mosquito morphogenesis, a novel function for POs. Herein we report the molecular cloning and characterization of *As-pro-PO III* gene and demonstrated that a putative *Drosophila* development regulatory motif, Zeste, positively regulates gene expression. Results of expression profile analysis and gene silencing assays implicate As-pro-PO III as a component in the morphogenesis in *Ar. subalbatus*.

2. Materials and methods

2.1. Biological materials

The source and maintenance of mosquitoes, *Ar. subalbatus*, and filarial worms, *Dirofilaria immitis*, were the same as previously described (Cho et al., 1998). Mosquito C6/36 cells were maintained in RPMI 1640 medium (GibcoBRL, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg streptomycin, and 25 μg/ml amphotericin B (Invitrogen, USA) at 27 °C.

2.2. RNA extraction and RACE-PCR

Except where otherwise noted, all DNA and RNA manipulations used standard techniques (Sambrook and Russell, 2001). Adult mosquito hemolymph were eluted using RNAzol™B (Tel-Test, Inc., USA) buffer, total RNA was extracted and with DNase treated. First strand cDNA was synthesized using the ReverAid™ H Minus First Strand cDNA Synthesis Kit (GibcoBRL, USA). Two degenerate primers were designed against the conserved copper-binding region (P-1, 5′-CAY CAY TGG CAY TGG CAY NTG-3′, and P-2, 5′-YTG NNA NCG YTA YAA NAN NGG-3′) from arthropod pro-POs (Fujimoto et al., 1995; Cho et al., 1998; Huang et al., 2001; Taft et al., 2001). cDNA was amplified by the following thermal cycle profile: 94 °C for 3 min and 30 cycles of 94 °C for 1.5 min, 50 °C for 2 min, and 72 °C for 10 min. RACE-PCR was carried out as described by Cho et al. (1998) with slight modifications. For 5′ RACE, the primer for first strand synthesis (P-3, 5′-AGA ACA GTT CAC CAC GGC GAT C-3′) was designed to overlap 80 bp at the 5′ end of the 600 bp fragment of pro-PO cDNA from *Ar. subalbatus*. The 5′ RACE fragment (650 bp) was amplified using P-4 (5′-TCC ACG AGG GCG TCC CAT GTG GTT TAC GGT-3′), and P-5 (5′–GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3′) primers. For 3′ RACE, the dT primer (P-6, 5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TT-3′) used for first strand cDNA synthesis, was designed to overlap the previous fragment 3′ end by 150 bp. The 3′ RACE fragment (1200 bp) was amplified using P-7 (5′-ATG GAA GTC TGC ATA ACT TTG-3′) and P-8 (5′-CUA CUA CUA CUA GGC CAC TCG ACT AGT AC-3′) primers. Primers P-9 (5′-CCG GAA TTC ATG GCC GAA AGA AAA TC-3′) and P-10 (5′-GCT CTA GAC TAT GTC CTG CTT ATG ATG-3′) designed from the 5′ and 3′ RACE fragment, were used to amplify full-length *As-pro-PO III* cDNA. The amplified fragment was verified by Southern blot hybridization and subcloned into the pGEM-T Easy vector (Promega, USA) for sequencing.

2.3. Genomic library screening and sequence analysis of As-pro-PO III

A lambda Dash II genomic library of *Ar. subalbatus* was screened with the *As-pro-PO III* cDNA clone as described by Sambrook and Russell (2001). The resulting positive genomic phage DNA was isolated for analysis by Southern blot, and subcloned into the pGEM-T Easy vector (Promega, USA) for sequencing. Sequence analysis and multiple alignments were performed using the NCBI database program (Altschul et al., 1990), and promoter region sequence was analyzed using TRANSFAC and GCG (University of Wisconsin, Genetic Computer Groups) software. The signal peptide was predicted using the SignalPv3.0 program.

2.4. Reverse transcription – PCR analysis of As-pro-PO III

RNA was extracted from pupae collected within 5 min after larval–pupal ecdysis, from pupae at different times following dsRNA or DEPC-water inoculation, and from adult female mosquitoes at various time intervals after microfilariae inoculation, blood feeding, bacteria inoculation, or HBSS buffer inoculation. The sequences of the specific primer were as follows: *As-pro-PO III*: P-11, 5′-ATG GAA GTC TGC ATA ACT TTG-3′, and P-12, 5′-TAC GCG AGC GGC GAT GCT GTT GA-3′; *As-pro-PO I*: P-13, 5′-ACT CAC CGA AGA AAA CGG CAC T-3′, and P-14, 5′-TTA TGT TCT AGC GAT CAC ACT-3′; *As-pro-PO IV*: P-15, 5′-CGT GGC ACC GTT CGA CTA TTC TTA-3′, and P-16, 5′-GCG TGC GTC AGG ATA CAA ACG ATC AC-3′; *As-pro-PO V*: P-17, 5′-GAT TTC CAT ATC TAG CTG GTT CTC-3′, and P-18, 5′-AAC TAC AGA ACC TTC TTC ACG CA-3′; and S7 ribosomal protein: P-19, 5′-TCC TGG AGC TGG AGA TGA AC-3′, and P-20, 5′-CCA GAT CCT CCA GGA TAG CA-3′. *As-pro-PO III* PCR was performed under the following conditions: 94 °C for 40 s, 60 °C for 40 s and 72 °C for 1 min for 35 cycles (but 58 °C extension temperature for other As-pro-POs). For S7 ribosomal protein fragment amplification, the number of PCR cycles was 25.

2.5. Luciferase reporter assay

The transfection and luciferase assay were carried as described by Zheng and Zheng (2002). The putative promoter region of *As-pro-PO III* gene (−1572 to +124 bp) and a series of deletion mutagenesis constructs of the proximal sequences were generated by PCR and subcloned into the pGL3 Luciferase Reporter Vector (promega, USA). Correct constructs were confirmed by sequencing. C6/36 cells were transfected using the transfection reagent TranIT-LT1 (Mirus, USA).

2.6. Electrophoretic mobility-shift assay (EMSA)

Nuclear cell extracts were prepared from 3-day-old females and the 4th instar larvae by NE-PER nuclear extraction reagent (Pierce, USA). Biotin end-labeled, double-stranded oligonucleotide containing the Zeste consensus sequence was generated by annealing with two single-stranded oligonucleotides; 5′-biotin-TGC ATA TGA TGA GCG CAA CAC AGT-3′ and 5′-ACT GTG TTG CGC TCA TCA TAT GCA-3′. For competition experiments, reactions were performed by adding unlabeled double-stranded Zeste or biotin end-labeled, non-specific, double-stranded oligonucleotide (5′-TGC ATA TGA GAC ATA CAA CAC AGT-3′). All reaction mixtures were electrophoresed on native polyacrylamide gel, transferred to a nylon membrane, and evaluated using a LightShift Chemiluminescent EMSA Kit (Pierce, USA) following the manufacturer's instructions.

2.7. Double-stranded RNA synthesis and inoculation

To specifically knockdown the expression of *As-pro-PO III* gene in *Ar. subalbatus*, a 716 DNA fragment (position −51 to +665) of *As-pro-PO III* gene was amplified by primer P-21 (5′-CAA TCT TCG ATT CAT TTA CAT CGT-3′) and P-22 (5′-CGG AGG TGC TCC CGC AGG GTA CA-3′), and the product was subcloned into the pBluescript II KS (+) vector and confirmed by sequencing. For single-strand RNA synthesis, plasmids were linearized with restriction enzyme, XhoI or SpeI, and *in vitro* transcribed using T3 or T7 Megascript transcription kits (Ambion, USA). The synthesized RNAs were adjusted to 1 μg/μl, and equal amounts of the complimentary, single strand