Published in final edited form as:

Dev Comp Immunol. 2018 October ; 87: 204–215. doi:10.1016/j.dci.2018.06.014.

Clip domain prophenoloxidase activating protease is required for *Ostrinia furnacalis* Guenée to defend against bacterial infection

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Abstract

The prophenoloxidase (PPO) activating system in insects plays an important role in defense against microbial invasion. In this paper, we identified a PPO activating protease (designated *OfPAP*) containing a 1,203 bp open reading frame encoding a 400-residue protein composed of two clip domains and a C-terminal serine protease domain from *Ostrinia furnacalis*. SignalP analysis revealed a putative signal peptide of 18 residues. The mature *OfPAP* was predicted to be 382 residues long with a calculated M_r of 44.8 kDa and pI of 6.66. Multiple sequence alignment and phylogenetic analysis indicated that *OfPAP* was orthologous to the PAPs in the other lepidopterans. A large increase of the transcript levels was observed in hemocytes at 4 h post injection (hpi) of killed *Bacillus subtilis*, whereas its level in integument increased continuously from 4 to 12 hpi in the challenged larvae and began to decline at 24 hpi. After *OfPAP* expression had been silenced, the median lethal time (LT_{50}) of *Escherichia coli*-infected larvae (1.0 day) became significantly lower than that of *E. coli*-infected wild-type (3.0 days, $p < 0.01$). A 3.5-fold increase in *E. coli* colony forming units occurred in larval hemolymph of the *OfPAP* knockdown larvae, as compared with that of the control larvae not injected with dsRNA. There were notable decreases in PO and IEARase activities in hemolymph of the *OfPAP* knockdown larvae. In summary, we have demonstrated that *OfPAP* is a component of the PPO activation system, likely by functioning as a PPO activating protease in *O. furnacalis* larvae.

Keywords

Insect immunity; Hemolymph protein; RNA interference; Melanization; Bacterial challenge

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1. Introduction

The innate immune system of insects defends the hosts from invading pathogens and parasitoids. This system includes cellular and humoral immunity. One immediate response is melanization catalyzed by phenoloxidase (PO). POs oxidize phenolic compounds to produce quinones implicated in hemolymph melanization, cuticle sclerotization, wound healing, and other immune responses (Gupta et al., 2005; Zhao et al., 2011). Foreign molecules can trigger the prophenoloxidase activation system (PPO-AS) that causes proteolytic cleavage of inactive prophenoloxidase (PPO) to form active PO. The prophenoloxidase activating protease (PAP), also known as prophenoloxidase activating enzyme (PPAE), is a key protease of the system (Satoh et al., 1999).

PAPs were first purified from cuticle of *Bombyx mori* larvae (Dohke, 1973), then identified and cloned from *Drosophila melanogaster* (Chosa et al., 1997), *Manduca sexta* (Jiang et al., 1998; Jiang et al., 2003a; Jiang et al., 2003b), *Holotrichia diomphalia* (Lee et al., 1998), *B. mori* (Satoh et al., 1999), *Pacifastacus leniusculus* (Wang et al., 2001), *Samia cynthia ricini* (Bao et al., 2007), *Spodoptera litura* (Arora et al., 2009) and *Plutella xylostella* (Shi et al., 2014). PAPs consist of one or two clip domains at the N-terminus and a serine protease catalytic domain at the C-terminus. The solution structure of *M. sexta* PAP2 dual clip domains suggests a potential PPO-binding site, a bacteria-interacting region, and a surface for activator or adaptor docking in each domain (Huang et al., 2007). In the absence of a protein cofactor from hemolymph, known as serine protease homologs (SPHs), the purified *M. sexta* PAP1, PAP2 and PAP3 do not efficiently generate active PO (Yu et al., 2003; Wang and Jiang, 2004). However, an SPH does not seem to be required for PAP-mediated activation of silkworm PPOs (Satoh et al., 1999). In *B. mori*, PPAEs are expressed in the fat body and then transported to the integument or hemocytes via the hemolymph for pupal cuticular melanization or the innate immune reaction (Zou et al., 2015). PAPs exist in hemolymph as zymogens at low concentration in larvae, and their expression in fat body is up-regulated after the challenge with bacteria (Jiang et al., 2010). In *P. xylostella*, abundance of *PxPAPa* mRNA significantly increased after treatment with *Micrococcus luteus* or *Escherichia coli* but not *Candida albicans*. *PxPAPb* was induced by *M. luteus* only. *PxPAP3* is differentially induced by *M. luteus*, *E. coli*, and *C. albicans* (Shi et al., 2014). The extensive expression of PAPs in various tissues from different insects suggests that PAPs may have broader functions in the immune process.

The host-parasite systems of lepidopteran larvae and parasitoid wasps have been explored in details. The Asian corn borer *Ostrinia furnacalis* is a serious agricultural pest in China. Previously, a prophenoloxidase cDNA (*OfPPO*) was cloned from *O. furnacalis* in our laboratory, and we infer from time-course studies of *OfPPO* gene expression and PO enzymatic activity that *OfPPO* in the integument is released from hemocytes and that the *OfPPO* expression was influenced at the transcriptional, translational, and post-translational levels by parasitization of *Microcentrus cingulum* (Feng et al., 2011). We have isolated the full-length Serine protease inhibitor 1 (*Ofserpin1*) cDNA from *O. furnacalis*. The results suggested that *Ofserpin1* has serine protease inhibitory activity and is likely involved in the regulation of prophenoloxidase activation system in *O. furnacalis* (Zhang et al., 2016). Our

study also demonstrated that Of β GRP3 is one of PRR members involved the PPO-activating system in *O. furnacalis* larvae (Wu et al., 2018). We had purified PPO from hemolymph of *O. furnacalis* larvae (Feng et al., 2008). However, very little is known about the role of OfPAP in *O. furnacalis* immunity. Here we report the characterization of PAP in *O. furnacalis* larvae. Larvae with OfPAP silenced exhibited a higher mortality than the control. The activities of PO and IEARase in hemolymph were somehow down-regulated after dsRNA of OfPAP gene had been injected into the larvae. Specifically, in this study we cloned a full-length cDNA of OfPAP, examined the tissue distribution and temporal expression profiles of the gene after bacterial challenge, and elucidated OfPAP's role as a molecule which responds to bacterial infection and activates the *O. furnacalis* PPO system. Such study is helpful for understanding the innate immune system in this agricultural pest.

2. Material and methods

2.1. Insect rearing

O. furnacalis Guenée (Lepidoptera: Pyralidae) larvae were reared at 27±1 °C, RH >80% and a photoperiod of 16L: 8D. The larvae were fed on an artificial diet as described previously (Feng et al., 2011), and the larval instars were determined by measuring the head-width.

2.2. cDNA cloning of OfPAP and sequence analysis

Total RNA was isolated from the 5th instar larvae of *O. furnacalis* using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. First-strand cDNA synthesis was carried out using PrimeScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). cDNA quality was checked on a 1% agarose gel and quantified by spectrophotometry at A₂₆₀. The resulting cDNA solution was stored at -20 °C until further analysis.

Alignment of multiple PAP sequences from other lepidopterans was performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Based on sequences of the two highly conserved regions in PAPs, a pair of primers, m-OfPAP-F and m-OfPAP-R, was designed (Table 1). The thermal cycling conditions were 3 min initial denaturation at 94°C, 30 cycles of denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s and extension at 72°C for 45 s, followed by a 10 min final extension at 72°C. Reaction products were separated on an agarose gel and a 509 bp fragment was cloned into the T-vector (Takara, Tokyo, Japan) and then sequenced on an ABI 3730 sequencer (SeqGen Inc., Torrance, CA).

A full-length OfPAP cDNA was obtained by 3' and 5'-rapid amplification of cDNA ends (RACE) based on sequence of the cloned fragment. The 3'-RACE was performed using 3'-RACE-ready cDNA and gene-specific primers (3'GSP1, 3'GSP2) and 3'CDS (Table 1). The PCR was carried out according to the program of 94°C for 4 min, 34 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and then an extension of 72°C for 10 min. For 5'-RACE PCR, nested PCR strategy was employed to increase specificity using Takara 5'-Full RACE Kit (TaKaRa). The PCR amplification was performed using 5'-RACE-ready cDNA, outer primer, inner primer, 5'GSP1 and 5'GSP2 (Table 1). The PCR conditions were 94°C for 3 min, 36 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s, and then an extension of

72°C for 10 min. The full-length cDNA sequence of *OfPAP* was verified by PCR amplification and DNA sequencing with the primers of PAP-F and PAP-R (Table 1).

The PAP amino acid sequences of other species were retrieved from GenBank. The amino acid sequence of *OfPAP* was aligned with other insects' PAPs previously identified using Clustal Omega. Protein domain structure was predicted using BLASTP. SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) was used for signal peptide prediction. Molecular mass and isoelectric point of the mature protein were calculated using ExPASy (http://web.expasy.org/compute_pi/). Phylogenetic tree was constructed by the neighbor-joining method using MEGA7.0. The *Fenneropenaeus chinensis* PPO activating factor (PPAF) (GenBank accession number: JX644447) was used as an out-group to root the phylogeny. Reliability of the tree branching was assessed by a bootstrap re-sampling method with 1,000 replications.

2.3. Expression analysis

To examine tissue distribution of *OfPAP* transcripts in day 1, 5th instar larvae, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the *OfPAP* specific primers e-PAP-F and e-PAP-R (Table 1). The larval tissues such as hemocytes, fat body, midgut and integument were collected and homogenized in Trizol solution (Invitrogen, Shanghai, China) for total RNA isolation. The total RNA samples were treated with RNase-free DNase I (Promega) to remove genomic DNA. First-strand cDNA was synthesized from 1.0 µg of total RNA using PrimeScriptTM RT Reagent Kit (TaKaRa) according to the manufacturer's protocol. The *O. furnacalis* ribosomal protein L8 (*rpL8*) gene was used as an internal control in all the experiments as previously described (Feng et al., 2011). qRT-PCR was carried out on a Bio-Rad CFX96 Real Time Detection System (Bio-Rad, USA) in 20 µL reaction containing 1 µL of cDNA from each tissue, 10 µL of SSOfast SYBR Green Mix, 1.0 µL of each primer (20 pmol/L) and 7 µL ddH₂O. The qRT-PCR reaction conditions were an initial denaturation step for 3 min at 95°C, followed by 40 cycles of denaturation (95°C for 10 s) and annealing-extension (60.5°C for 10 s). *O. furnacalis* *rpL8* primers *rpL8-F* and *rpL8-R* (Table 1) were used to calibrate the cDNA template as an internal control under the same cycling conditions. All samples were analyzed in biological triplicate in three independent experiments performed using different tissues from *O. furnacalis* larvae. The comparative CT method (2^{-CT} method) was used to analyze the expression level of *OfPAP* (Livak and Schmittgen, 2001).

To analyze the temporal and spatial expression pattern of *OfPAP* in larvae after an immune challenge, day 1, 5th instar larvae were divided into four groups: killed *E. coli*-challenged, killed *Bacillus subtilis*-challenged, untreated group and injected with sterile saline (0.65% NaCl, 0.025% KCl, 0.03% CaCl₂, 0.025% NHCO₃, pH6.8). To induce the larvae with *E. coli* or *B. subtilis*, 4 µL of 3×10⁶ CFU of *E. coli* BL21 (DE3) or *B. subtilis* in 0.85% (w/v) NaCl were injected into larvae. The control larvae were injected with 4 µL the saline or without injection. At 2, 4, 6, 8, 10, 12, 24 and 36 h post injection (hpi), hemocytes, fat body, and integument from ten individual larvae were collected into CAC buffer (100 mM CaCl₂, 10 mM sodium cacodylate, pH 6.5) as an anticoagulant.

2.4. RNA interference (RNAi)

Double-stranded (ds) RNAs of *OfPAP* and green fluorescent protein (GFP) were prepared by following the protocol of RiboMax™ T7 system (Promega, USA). A template for *in vitro* transcription reactions was prepared by PCR amplification of a clone containing the *OfPAP* ORF using gene-specific primers (Table 1) with a T7 polymerase binding sequence at 5' end. After phenol-chloroform extraction and heat treatment, the dsRNA was diluted with nuclease-free water to a final concentration of 5 µg/µL for injection into 4th instar larvae. *GFP* dsRNA and nuclease-free DEPC water were served as negative controls. Fourth instar larvae were injected intra-abdominally with 20 µg of *OfPAP* dsRNA (RNAi group). The GFP control group received a 4 µL injection with 20 µg of *GFP* dsRNA. The third group of larvae received a 4 µL injection of nuclease-free DEPC water to mimic the handling effect. Sixty 4th instar larvae were treated in three independent experiments, respectively. The puncture was sealed immediately with liquid paraffin. Larvae were removed from the slide after injection and raised at 25°C until analysis. qRT-PCR was performed to confirm the efficacy of RNAi as described above. All reactions were completed in triplicate using independently extracted RNA samples.

2.5. Assessment of efficacy of RNAi and cumulative mortality after RNAi

For cumulative mortality analysis after *OfPAP* RNAi, following injection of *OfPAP* dsRNA, 20 larvae were inoculated with 3×10^6 CFU of live *E. coli* BL21 (DE3) intra-abdominally (RNAi-infected). Twenty larvae were treated with *OfPAP* RNAi without bacterial injection (RNAi) and 20 larvae were inoculated with 3×10^6 CFU of *E. coli* BL21 (DE3) only (infected). The control group received a 4 µL injection of *GFP* dsRNA (GFP). The numbers of dead larvae were recorded at the same time each day for each group over 8 days post injection, and the mortality was calculated from three independent experiments.

For assessment of the efficacy of RNAi in the larvae hemocytes and integument, the expression levels of *OfPAP* gene were measured by qRT-PCR after 3 days post injection of *OfPAP* dsRNA. *GFP* dsRNA and nuclease-free DEPC water were served as negative controls. The biological experiments were conducted in triplicate and qRT-PCR was repeated three times for each sample. Larval hemolymph was collected at 48, 60, 72, 96, 120 h post injection after RNAi (at least 20 larvae per time-point for experimental samples and controls). Hemolymph collected by capillary suction from cut pro-legs of larval abdomen was used for measuring PO and IEARase activities.

2.6 Total hemolymph viable bacterial levels in *OfPAP* dsRNA silenced larvae

To test possible involvement of the *OfPAP* in bacterial clearance, 2×10^5 CFUs of *E. coli* isolate were intra-abdominally injected into the *OfPAP* dsRNA knockdown larvae at 24 h after the first dsRNA injection, and then hemolymph samples were collected individually from larvae at 6 h post *E. coli* challenge and serially diluted in sterile phosphate buffer saline (pH 7.4). Each dilution of the hemolymph was then spread onto an LB-agar plate (10 µL/dot) and further incubated at 30°C overnight followed by counting of *E. coli* colonies. All experiments were conducted in triplicate.

2.7. Activities of PO and IEARase from hemolymph of larvae after *OfPAP* RNAi

To investigate involvement of *OfPAP* in larval PPO system, the activities of PO and IEARase in 100 samples of hemolymph were measured after *OfPAP* RNAi at different time post injection. Hemolymph samples (approximately 30 μ L/larva) were collected into chilled polypropylene tubes as described above from cut prolegs of fifth instar larvae after *OfPAP* RNAi. Hemocytes were pelleted by centrifugation at $800\times g$ for 15 min. Aliquots (10 μ L) of fresh plasma (*i.e.* cell-free hemolymph) were mixed with 150 μ L 2 mM dopamine in 50 mM sodium phosphate, pH 6.5, and placed in a microplate reader for measuring PO activity in a kinetic mode. One unit of PO was defined as 0.001 absorbance increases at 470 nm per min. The enzymatic reactions were carried out in triplicate.

IEARase activity in the cell-free hemolymph samples (30 μ L) was assayed using 200 μ L of 50 μ M acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR p Na, Sigma-Aldrich A0180) as a chromogenic substrate in 100 mM sodium phosphate buffer, pH 8.0 (Jiang et al., 2003b). The reactions were carried out at 30 $^{\circ}$ C for 5 min and the release of *p*-nitroanilide was measured spectrophotometrically at 405 nm. One unit of IEARase activity was defined as 0.001 absorbance increase at 405 nm per min. The enzymatic reactions were carried out in triplicate.

2.8 Protein determination

Protein concentration in samples was measured by a dye-binding assay (Bradford, 1976). Standard curves were constructed using bovine serum albumin.

2.9. Data analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by LSD test. The Kaplan-Meier survival analysis was conducted with Statistical Package for the Social Sciences software (SPSS version 23.0). Differences with a *p*-value less than 0.05 were considered statistically significant for all treatments.

3. Results

3.1. Molecular characteristics of the full-length *OfPAP* cDNA and protein

By using a degenerate primer pair designed on the basis of conserved regions in the PAPs reported in several insects, we obtained a 509 bp cDNA fragment encoding a putative PAP from *O. furnacalis* larvae. The full-length cDNA of *OfPAP* (GenBank accession number: GU229936), identified using 5' and 3' RACE, consists of 1,455 nucleotides, including an open reading frame (ORF) of 1,203 bp that encodes a 400-residue protein (Fig. 1A). The cDNA has a 5' untranslated region (UTR) of 66 nucleotides; a 3' UTR of 189 nucleotides including a putative polyadenylation signal (AATAAA) and a poly (A) tail. SignalP analysis revealed a putative signal peptide of 18 residues. The predicted mature protein (382 residues) has a calculated molecular mass of 44.8 kDa and a theoretical isoelectric point of 6.66. No potential N-linked glycosylation sites were identified. *OfPAP* contains two clip domains (residues 22-70 and 76-118) (Figs. 1B and 2). The conserved SP catalytic domain (residues 152-394) was also identified by BLASTP. Multiple amino acid sequence alignment of the *OfPAP* with the other insect PAPs revealed that *OfPAP* has the common structural

features of the clip-domain serine protease (SP) family. The first clip domain contains six conserved Cys residues at positions 22, 28, 34, 60, 69 and 70 but the second clip domain only have four of the six Cys residues at positions 76, 86, 109 and 118 (Fig. 1B). Lys⁹¹ and Tyr¹¹⁹ substitute the conserved Cys pair that forms a disulfide bond in *M. sexta* PAP2 (Huang et al, 2007), and the complete missing of 2nd clip domain was found in *P. xylostella* PAP3a. Three conserved H¹⁹⁴, D²⁵⁸ and S³⁵⁷ form a catalytic triad in the C-terminal SP domain (Fig. 2).

A phylogenetic tree was constructed using the neighbor-joining method based on PAP/PPAE/PPAF sequences (Fig. 3), with the putative PPAF of *F. chinensis* acting as the out-group. The nineteen PAPs/PPAEs/PPAFs were divided into two main branches: one mainly consisting of insect PAP3's and the other consisting of insect PAP1's. BLASTP results indicated that the deduced amino acid sequence of *OfPAP* has significant similarity with the other PAPs or PPAFs, such as 40% with PAP3 from *M. sexta*, 38% with PAP3 from *P. xylostella*, 37% with PPAE3 from *S. litura*, and 36% with PPAE3 from *Danaus plexippus*. Note that *P. xylostella* PAP3a is similar in size to *OfPAP*, lacks the second clip domain, contains the conserved Cys¹³⁰ and Cys¹⁴²Cys¹⁴³ in the linker region (Fig. 1). We found *OfPAP* containing one typical clip domain and one atypical clip domain was between *P. xylostella* PAP3a which had one clip domain and *M. sexta* PAP2 and *M. sexta* PAP3 which had two clip domains in the phylogenetic tree (Fig. 3). Therefore, we infer *OfPAP* may be the essential evolutionary hub for Clip SP from one clip domain to two clip domains.

3.2. Differential expression of *OfPAP* gene in the tissues

qRT-PCR was employed to determine the levels of *OfPAP* mRNA in different tissues of *O. furnacalis* larvae, with the *rpL8* used as an internal control for normalizing the RNA samples. The result indicated that the mRNA of *OfPAP* was detected in hemocytes, fat body, midgut and integument. The transcript levels of *OfPAP* were low to moderate in integument and fat body. The highest and lowest levels of *OfPAP* mRNA were observed in hemocytes and midgut (Fig. 4), respectively.

3.3. Temporal and spatial expression pattern of *OfPAP* in response to a bacterial infection

To further study the expression of *OfPAP* during immune response in *O. furnacalis* larvae, hemocytes, fat body and integument were collected at different time points after a bacterial challenge. The *OfPAP* mRNA level was measured using qRT-PCR method. In hemocytes, a dramatic increase in the levels of *OfPAP* transcripts in *B. subtilis*-challenged larvae was observed at 4 hpi, which was 13.5-fold higher than that in the control group of saline injection. The expression levels of *E. coli*-challenged group were not significantly different to the untreated group from 8 to 36 hpi (Fig. 5A). In fat body, the expression of *OfPAP* in *B. subtilis*-challenged group was notably higher than those in saline injection group with the peak level of 5.2-fold at 24 hpi. The mRNA levels of *OfPAP* in *E. coli*-challenged group were not significantly different from the saline injection group ($p > 0.05$) (Fig. 5B). In integument, the *OfPAP* expression of *B. subtilis*-challenged larvae continuously increased from 4 to 12 hpi, and then began to decline at 24 hpi (Fig. 5C). The mRNA levels in *B. subtilis*-challenged larvae arose to 3.8 ± 0.5 , 10.9 ± 1.7 , 28.5 ± 1.9 , and 10.5 ± 0.1 fold of those to the saline control at 8, 10, 12 and 24 hpi, respectively. In comparison, the mRNA

levels of *OfPAP* in *E. coli*-challenged group was 5.7 ± 0.6 , 3.5 ± 0.4 , 3.8 ± 0.3 , and 2.0 ± 0.3 fold higher than the saline group at 8, 10, 12 and 24 hpi, respectively.

3.4. Effect of *OfPAP* gene silencing on mortality of *O. furnacalis* larvae

Effects of the knockdown of *OfPAP* transcription in larvae were investigated after dsRNA injection. To perform RNAi, naïve 4th instar larvae of *O. furnacalis* were injected with *OfPAP* or *GFP* dsRNA (20 µg/larva, 60 larvae) or DEPC-treated water (4 µL/larvae, 60 larvae). As compared with the *GFP* dsRNA injection group, mRNA levels of *OfPAP* in hemocytes was reduced to approximately 50% at 60 hpi and 36% at 72 hpi (Fig. 6A). In integument, transcription of *OfPAP* was reduced to approximately 26% at 60 hpi and 24% at 72 hpi (Fig. 6B). While *OfPAP* mRNA levels had no significant changes in the DEPC-H₂O and *GFP* dsRNA injection group. Those data indicated that dsRNA-mediated knockdown of *OfPAP* transcription was specific and successful in *O. furnacalis* larvae.

The role of *OfPAP* in resistance against bacterial pathogens was investigated by measuring survival rates after RNAi-mediated silencing of *OfPAP* in larvae. The Kaplan-Meier survival analysis showed that the median lethal time (LT₅₀) of *GFP*-dsRNA injection group was 4.0 days and that of *E. coli*-infected wild-type larvae was 3.0 days, significantly longer than that of *E. coli*-infected *OfPAP*-RNAi larvae (1.0 days) ($p < 0.01$) (Fig. 6C). These data suggested that *OfPAP* was required for resistance against infection by bacterial pathogens.

3.5. Increase in counts of live *E. coli* in hemolymph of *OfPAP* knockdown larvae

To elucidate *OfPAP*'s role in the larval defense against *E. coli* infection, live *E. coli* cells were injected into the *ofPAP* dsRNA knockdown larvae at 24 h after the first dsRNA injection. The CFUs of *E. coli* in the *OfPAP* dsRNA-treated larval hemolymph were 3.5-fold higher than that in the control larvae injected with *GFP* dsRNA (Fig. 7). Thus, *OfPAP* seems to participate in the larval PPO system and play an essential role in protecting larvae from *E. coli* infection.

3.6. Reduction of PO and IEARase activities in hemolymph after *OfPAP* RNAi

To test possible involvement of *OfPAP* gene product in the *O. furnacalis* larval PPO activation system, we collected hemolymph from the *OfPAP* dsRNA-treated larvae at different time points after injection and measured the PO and IEARase activities. *M. sexta* PAPs efficiently hydrolyzes acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (Jiang et al., 2003a). There was a remarkable reduction in PO activity, down to 59.1% of the control (*GFP* dsRNA injection) at 120 hpi. No significant difference in the PO activity was observed between the *GFP* dsRNA and DEPC-H₂O groups (Fig. 8A). The IEARase activity displayed a significant decrease in the *OfPAP* knockdown larvae (Fig. 8B). Combined with the involvement of *OfPAP* in bacteria elimination, we inferred that *OfPAP* was capable to kill invading bacteria likely through PPO activation cascade. *OfPAP* is likely to be a key member of the PPO activation cascade and may function as *O. furnacalis* PPO activating protease.

4. Discussion

Melanization through the activation of the PPO activating system is an essential immune reaction against microbial or parasitic infections in the arthropods (Cerenius et al., 2008; Charoensapsri et al., 2011). An endogenous activation system activates PPOs in insects and crustaceans, and PO activity was often measured to monitor PPO activation (Söderhäll and Häll, 1984; Perazzolo and Barracco, 1997). PPO is synthesized as a zymogen and stored in oenocytoids in *O. furnacalis* larvae (Feng et al., 2008). However, there is little knowledge about the activation and regulation of OfPAP involved in the *O. furnacalis* PPO activation pathway. In this study, we cloned the *OfPAP* cDNA from the larvae and examined its expression patterns in tissues before and after a bacterial challenge.

Clip-domain serine proteases (CLIPs) are vital components of extracellular signaling cascades in various biological processes in invertebrates (Kanost and Jiang, 2015). Many studies have demonstrated that clip-domain SP-SPH systems function in the innate immune response of insects and crustaceans (Cerenius et al., 2010). A large number of CLIPs are discovered in arthropods after an SPH was characterized in *D. melanogaster* (Murugasu-Oei et al., 1995; Lu et al., 2014a; Lu et al., 2014b). CLIPs were also identified in the crab *Eriocheir sinensis* (Jia et al., 2018). A clip-domain and a trypsin-like serine protease domain were also identified in pearl oyster *Pinctada fucata* (Zhang et al., 2009). *M. sexta* PAP2 and PAP3 contain two N-terminal clip domains followed by a C-terminal catalytic domain, whereas *M. sexta* PAP1 contains only one clip domain at its N-terminus (Jiang et al., 2003a). In the present study, the clip-domain SP, designated as *OfPAP*, contains one typical and one atypical clip domain at the N-terminus and a catalytic domain at the C-terminus. Folding of typical clip domains is stabilized by three disulfide bridges formed between highly conserved Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6. In the second clip domain of *OfPAP*, the third bond is lost due to the substitution of both Cys3 and Cys6 by Lys⁹¹ and Tyr¹¹⁹ (Fig. 1). It is unclear how stable the domain is but *OfPAP* appears to be functional, according to the results of the RNAi tests. More direct evidence is needed to prove that the atypical clip domain does not severely impair the molecular function. *P. xylostella* PAP3a, located near bottom of the branch of the dual clip-domain SPs (Fig. 3), has a typical clip-1, a Cys-free region similar in size to *OfPAP* clip-2, and a linker containing Cys¹²⁶ and Cys¹³⁹Cys¹⁴⁰. These data suggest clip-1 is more important than clip-2. So far, the low number of structures resolved by crystallography limits our knowledge of the clip-SPs folding. In further investigation, suitable cryo-conditions of the crystal of *OfPAP* will be explored, and the high resolution diffraction data using multiple-wavelength anomalous dispersion (MAD) and multiple isomorphous replacement (MIR) approaches could be collected, which might enable us to determine the final structure of clips in *OfPAP*. The detailed structure will provide useful information to elucidate the biological function of *OfPAP*.

OfPAP belongs to the B2 subfamily of CLIPs, whose C-terminal catalytic domain has an extra pair of Cys residues once considered to a signature of PAPs (Kanost and Jiang, 2015). While some CLIPs do not activate PPO, most of them do participate in innate immune responses. Whether *OfPAP* are the terminal clip-SP that directly cleaves PPO still remains to be further studied. The biochemical analyses, such as direct activation of downstream proteins will be carried out using purified proteins *OfPAP* to show *OfPAP* indeed activates

PPO by cleavage of OfPPO. The investigation of the substrate (s) for OfPAP *in vivo* will also be essential for resolving the melanization pathway in *O. furnacalis*. The phylogenetic analysis demonstrates its close relationship with the known PAPs/PPAEs/PPAFs (Fig. 3). Its amino acid sequence is 40% identical to *M. sexta* PAP3, a terminal enzyme in the PPO activation cascade. *S. ricini* PAP shares the same domain structure with *M. sexta* PAP2, PAP3 and *B. mori* PPAE, which have two N-terminal clip domains and a C-terminal SP domain (Bao et al., 2007). Based on that, we suspect *OfPAP* acts as a PAP in the PPO activation pathway.

The expression pattern of the OfPAP in tissues was somewhat different from that of PAPs in other animals. CLIPs are often expressed in insect hemocytes owing to their roles in innate immune response (Kanost and Jiang, 2015). Our tissue distribution analysis by qRT-PCR revealed that *OfPAP* transcript level was high in hemocytes, which suggest that OfPAP may be involved in cellular melanization. This result is in accord with an observation in the crayfish, *P. leniusculus*, whose PPAE mRNA were detected in hemocytes (Wang et al., 2001). In the shrimp *Litopenaeus vannamei*, the transcription of *IvPPAE1* in gill was in fact due to the fixed hemocytes (GH). The mRNA level in these hemocytes showed a different regulation pattern than hemocytes in circulation (Jang et al., 2011). We suspected that hemocytes were still the center for production of *OfPAP*. The OfPAP expression in integument after *B. subtilis* challenge seemed to show stronger response than that in hemocytes, which suggest that OfPAP protects against pathogen invasion via the integument. Sclerotized cuticle presents a barrier to infection, and melanization around pathogens is helpful to kill the pathogens. In *B. mori*, the cuticle of larvae contains PPO and its activating system (Ashida and Brey, 1995) and the PPAE mRNA level in the larval integument is much higher than that in hemocytes or fat body (Sato et al., 1999). In *S. ricini*, PAP gene transcripts were barely detected in naive fat body (Bao et al., 2007). In the shrimp *Penaeus monodon*, *PmPPAE1* mRNA is restricted to hemocytes (Charoensapsri et al., 2009). In the moth, *Ofserpin1* mRNA and protein were detected in all the tested tissues, particularly in hemocytes and integument. (Zhang et al., 2016.), and *OfPAP* transcripts are detected in larval hemocytes, fat body and integument (Fig. 4). Most arthropods live in natural habitats in the presence of many microbes. The infection by natural microorganisms invading through the integuments is common. We suppose that the expression and localization pattern of these enzymes may be related to the function and activation of PPO.

The *IvPPAE1* was not simply down-regulated by *Vibrio alginolyticus* challenge but also up-regulated in the site of bacteria clearance. The expression feature was termed “selectively enhanced expression” (Jang et al., 2011). The current data showed the *OfPAP* transcript level was up-regulated from 8 h to 24 h after *B. subtilis* challenge in fat body and integument. However, in all studied crustaceans, their PPAE mRNA levels were either not affected or down-regulated within 48 h (Liu et al., 2007). But after bacterial infections, *OfPAP* were significantly upregulated during the early stage of bacterial infection in hemocytes, while *OfPAP* were mainly upregulated during the late stage of bacterial infection in fat body and integument (Fig. 5). Those difference may result from *OfPAP* was an inducible protease, so that slight upregulation of *OfPAP* in fat body and integument seemed to show stronger responses.

Double stranded RNA (dsRNA)-mediated RNA interference is an efficient technique for gene function testing. To further understand the role of *OfPAP*, we down-regulated the gene expression via dsRNA injection, and monitored the effect on the hemolymph PO and IEARase activities. Our RNAi experiment showed *OfPAP* silencing significantly decreased the PO and IEARase activities in comparison to the control group, suggesting that *OfPAP* is a protease required for *O. furnacalis* PPO activation. Likewise, the RNAi approach in *A. gambiae* established the function of the mosquito *CLIPB4*, *CLIPB8* and *CLIPB14* as components of the PPO activation system (Paskewitz et al., 2006). Similarly, functions of the two CLIP genes from *P. monodon* (*PmPPAE1* and *PmClipSP1*) were found to be different using RNAi tests. *PmPPAE1* participates in the PPO system and other immune responses in the shrimp (Charoensapsri et al., 2009). *PmClipSP1* is only involved in the latter (Amparyup et al., 2010). Additionally, the cumulative mortality in *OfPAP* knockdown larvae after the systemic challenge with *E. coli*, was significantly increased when compared to the control larvae. These observations are similar to those previously reported in the *PmPPAE1* knockdown *P. monodon* shrimps (Charoensapsri et al., 2009). Therefore, we suggest that *OfPAP* is required to activate the PPO pathway and also plays a crucial role in the *O. furnacalis* immune defense against *E. coli* infection.

Most results suggest that PAPs cleaved PPO to activate PPO cascade, but there are still many novel findings indicated the complex mechanisms of PPO cascade. Several clip-domain SPs have been shown to participate in the PPO activation and Toll signaling pathways in insects and crustaceans (Amparyup et al., 2012; Gorman et al., 2007). Recombinant *O. furnacalis* SP1 was found to activate proSP13 to directly cleave PPO and then activate PPO cascade in the corn borer's plasma (Chu et al., 2015). Additionally, SP105 in *O. furnacalis* directly cleaved and activated PPO cascade and therefore acted as the prophenoloxidase-activating protease (Chu et al., 2017). All of them suggested that they were required for the activation of PPO cascade, and SP13 and SP105 were capable to directly cleave PPO. Despite structural differences, PPAEs in crustaceans by themselves activate PPO (Nakamura et al., 1985), unlike the insect PAPs that need cofactors to active PPOs (Hoffmann, 2003). The requirement of proteins other than PAPs for PPO activation was observed in *Hyalophora cecropia*, *M. sexta*, *H. diomphalia*, and *T. molitor*, but not in *B. mori* (Andersson et al., 1989; Wang and Jiang, 2004; Lee et al., 1998; Lee et al., 2002; Satoh et al., 1999). *H. diomphalia* PPO-activating factor I (PPAF-I) cleaved PPO polypeptide-1 and -2 between Arg⁵⁰ and Arg⁵¹, respectively, and generated little PO activity. In the presence of cleaved *H. diomphalia* PPAF-II (a clip-domain SPH), the precursor of PPAF-II is activated by another clip-domain SP, *H. diomphalia* PPAF-III (Kim et al., 2002). Some results suggest that the PPO cascade are also activated by ethanol and cetylpyridinium chloride (Lu et al., 2014b; Wu et al., 2018). The PPO cascade in insects are not only regulated by insect immune system, but also undergo the suppression of pathogenic secreted protein. Reeler was involved in the PPO activation system in *B. mori* (Bao et al., 2008), and some proteins produced by pathogens often inhibit the melanization in host insects, such as *Microplitis demolitor* can use bracovirus to produce Egf1.0 which can block the formation of high mass complex (~670 kDa) primarily associated with melanization activity in *B. mori* to inhibit the PPO cascade (Beck and Strand, 2007; Clark and Strand, 2013). The number of PPO genes ranges from one to ten in insects (Lu et al., 2014). In *Drosophila*, there are three PPO genes,

and PPO1 and PPO2 produced by crystal cells are responsible for most melanization in hemolymph, and PPO3 expressed in lamellocytes and is involved in the melanization in the process of encapsulation (Liu et al., 2012). However, the functions and localizations of the four PPO genes identified in *O. furnacalis* are still unclear (Feng et al., 2011; Zhang et al., 2016). In addition, some microRNAs identified in *M. sexta* may be involved in the regulation of PPO cascade (Zhang et al., 2014).

Taken together, the activation system of PPO cascade is more complex than that we have known, and there is a long way to decipher the PPO activation system except for the PAP investigated in this study. We have identified a novel clip-SP, named *OfPAP* from *O. furnacalis* larvae, and suggested its function in the larval PPO activation system as well as a role in the *O. furnacalis* defense against bacterial infection. Further biochemical characterization is important for a better understanding of this and other proteases in the pest PPO activation system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by National Natural Science Foundation of China Grants 31171895 and 31471817 (to C. Feng) and National Institutes of Health Grants GM58634 and AI112662 (to H. Jiang).

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Highlights

- cDNA isolation of *Ostrinia furnacalis* PAP.
- A large increase of *OfPAP* mRNA in hemocytes at 4 h post injection of killed *Bacillus subtilis*.
- Notably lower LT_{50} of *OfPAP*-RNAi larvae than that after *Escherichia coli* infection.
- Significant reduction in PO and IEARase activities of hemolymph of the *OfPAP* knockdown larvae.

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1  gaaaatatagttattatcagcgggctgcagagcagtcgcgttgcagcgtccagtgaa
61  ctgaacatgaagtggactcttttgattggtctcttggcattcgttagccacctgcatgca
    M K W T L L I G L L A F V S H L H A
121 aacgaggaatgccctgctggcgagacatgtatgtcaataaaggaaatgcgcgaagatatt
    N E E [C P A G E T C M S I K E C P Q D I
181 acggacttggctagaatgagaccgaagccgaagatatattagaagactaaagaaagct
    T D L A R M R P K P K D I L E R L K K A
241 cgatcgggtaaacggctgacaaaaactttgtgtaaaacgcgcagagtggttacaca
    R C G K P A D K K L C Q J K T P P E I C Y I
301 tgggacggacgacgggggtgtgtgaattgtatgacaaagaacgactaaaaatataat
    W D G R P G V C E L Y D K E R T K K L N
361 atgacaccttggaaaataaaatgtcaaatgcataataaaatcgactgatatctgc
    M T P W K I K M S K C I N K N R L D I C I
421 tacagtaacgagcctgagcggcaacacgctaattgcgagagcgcttccggctagctta
    Y S N E P E R Q H A N C E S A F P A S L
481 gactcggatgctgtggaatcgagaaaatgcaggagacagagattatggggagggacggc
    D S G C C G I E K S A G D R I M G G T A
541 accaagatcgatcagttcccggtggcgtgttgcataatccaaagccctccatgtcc
    T K I D Q F P W L A L L K Y P K P S M S
601 tgcagtggtgacttataagcagcaaatcgtgttaacagctgccactgcgtagacaa
    C S G A L I S S K Y V L T A A H C V D N
661 gctgacaaggaccaagcaacgcgctgtgtacttggggaatacgaagagtactaactat
    A D K D Q A T D V V L G E Y D K S T N Y
721 gactgtgacgatgagctctgcatcgaaaatgccactgtcaccgccaagattctgcaatc
    D C D D E L C I E N A T V T A K I L Q I
781 ataatacaccgcgatttgcacaaatccataagaattacgacatcgctctcattagactg
    I I H P D F D Q I H K N Y D I A L I R L
841 gagaaagcgggtgcatttacagatttcataacgcgatatgtcttcgagccacgttgc
    E K A V P F T D F I Q P I C L P S H V A
901 gatttgactaaaaatccacagaaaagtccaacttagtggtcgccgggtggggagagctc
    D L T K N P P E K F K L V V A G W G D V
961 gatccgaatcaccatcactcttctaagtccaagctccacgttcaactgcgcttgcgat
    D P N H H H S S N V K L H V Q L P F V D
1021 gcacttgcatgtcgccgaactgtaagagagttgtggacaagtcagatctgcgcggcgcc
    A L A C R R T V R E L W T S Q I C A G G
1081 gagggcaaaaaggactcttgcaacggcgattccggcgccgcctcatgtacgaaaggag
    E A K K D S C N G D S G G P L M Y E R E
1141 gactcgtctttgaagtcctcgcatcaccagctacacgagagagaagtgtggcactgcg
    D V V F E V L G I T S Y S E E K C G T A
1201 aacgtaccgcgatatatactaaagtccattcgtatatgaactggatacattttaagagt
    N V P A I Y T K V H S Y M N W I H F K S
1261 aggcgtaaccctctaactacgacggagatttggacgtgcgaatttttaattcacaat
    R P *
1321 gactaagtaagtacagaattttaatgacacacatgtatgttccaaatagtaggtataaca
1381 tacaattatatttttaattatctgttacattgtatagcagttattagtgcttacata
1441 aatctgtacaccagaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa

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(A)

(B)

Fig. 1. Nucleotide and deduced amino acid sequences and predicted domain structure of *O. furnacalis* PAP.

(A) The sequence is deposited in GenBank (accession number: GU229936). Nucleotides are numbered from the first one at the 5'-end. The stop codon is marked with an asterisk (*). The polyadenylation signal (AATAAA) and poly (A) tail are marked with short dash lines. The signal peptide sequence is shaded gray. The two clip domains are included in parentheses and highlighted with a wavy line. The SP catalytic triad (H, D, S) at the active site are indicated with "@". Conserved chymotrypsin-like SP catalytic domain is underlined.

(B) Predicted domain structure of *OfPAP* by BLASTP.

Fig. 2. Sequence alignment of PAPs from lepidopteran insects.

OfPAP is compared with *Danaus plexippus plexippus* PPAE3 (OWR45231), *M. sexta* PAP3 (AAX18637), *S. litura* PPAE3 (AAW24481) and *P. xylostella* PAP3a (ANH58162). The ClustalO program was used for alignments. The predicted disulfide linkages between conserved cysteines of the clip domains are shown with yellow lines, respectively. Amino acid residues constituting the catalytic triad (His, Asp and Ser) are shaded pink.

Fig. 4. Tissue-specific expression of the *OfPAP* mRNA in larvae measured by SYBR green qRT-PCR.

The tissues include hemocytes, fat body, midgut and integument collected from day 1, 5th instar larvae. Relative RNA levels are shown as mean ± SD (n=3).

(A)

(B)

(C)

Fig. 5. Expression profiles of *OfPAP* in hemocytes (A), fat body (B) and integument (C) of the 5th instar naïve larvae or larvae after a *B. subtilis* or *E. coli* challenge at different times.

The *O. furnacalis rpL8* gene was used as an internal control. Vertical bar represents the mean $\pm$ SD (n=3). Significant differences were indicated with “*” at the $p < 0.05$ level, “***” at the $p < 0.01$ level and “****” at the $p < 0.001$ level, respectively. The same below.

(C)

Fig. 6. Effectiveness of dsRNA-mediated knockdown of *OfPAP* expression and its impact on survival of *O. furnacalis* larvae upon bacterial infection.

Effect of dsRNA injection on *OfPAP* transcript levels in hemocytes (A) and integument (B) at different times. Levels of *OfPAP* mRNA in relative to *rpL8* were determined by qRT-PCR. (C) Survival test of naïve and *OfPAP* dsRNA-treated larvae. After injection of *OfPAP* dsRNA, twenty larvae were inoculated with *E. coli* intra-abdominally (RNAi-infected). Twenty larvae were treated with *OfPAP* RNAi without bacterial infection (RNAi) and 20 larvae were inoculated with *E. coli* only (infected). Twenty naïve larvae injected with dsRNA specific to green fluorescent protein (*GFP* dsRNA) were used as controls. The number of dead larvae was counted daily. Results are expressed as mean $\pm$ SD of three independent experiments.

Fig. 7. Number of viable bacteria (colony forming units or CFU) in hemolymph of *OfPAP*-silenced larvae after a systemic *E. coli* infection.

Larvae were challenged with *E. coli* (2×10^5 CFUs) following the injection of *OfPAP* dsRNA. Control larvae were injected with GFP dsRNA or saline solution only. The bacterial CFUs in hemolymph from the knockdown larvae were determined at 6 h after *E. coli* infection by a modified total plate count method and are shown as mean $\pm$ SD from three independent experiments. Asterisks indicate significant difference ($p < 0.05$).

A

B

Fig. 8. Time-dependent changes in PO (A) and IEARase (B) activities of larval hemolymph after RNAi-mediated silencing of *OfPAP* expression.

Activities are shown as mean $\pm$ SD (n=3) from three independent experiments.

Table 1.

Nucleotide sequences of the PCR primers

Name	Purpose	Sequence (5'–3')
m-OfPAP-F	middle fragment cloning	ACTGCGYCMCAYTGYGT
m-OfPAP-R		CATKAGVGSDDCCCKGAGTC
3'GSP1	3'-RACE PCR	TGCTGCCCATTGCGTAGA
3'GSP2		CACTGTCCACGCCAAGAT
3'CDS		ATTCTAGAGGCCGAGGCGGCCGACATG-d (T) 30VN
5'GSP1	5'-RACE PCR	AGGCTCGTTACTGTAGCAGA
5'GSP2		AGCCGGTTTACCGCATCG
5'GSP3		TTTCGGCTTCGGTCTCAT
5'Outer primer		CATGGCTACATGCTGACAGCCTA
5'Inner primer		CGCGGATCCACAGCCTACTGATGATCAGTCGATG
PAP-F	full length cDNA identification	GAAAATATAGTTATTATCAG
PAP-R		CTGGTTGTACAGATTTATTGTAAGCAC
e-PAP-F	PAP qRT-PCR	CTGTCCACGCCAAGATTC
e-PAP-R		CACCGCTTTCTCCAGTC
rpL8-F		ACGGAGGTGGTAACCATCAACA
rpL8-R		ACGCCTCCTTCTTGGTGTCG
	gene silencing	
dsPAP(T7)-F		GGATCCTAATACGACTCACTATAGGGAAGTGGACTCTTTGAT
dsPAP(T7)-R		GGATCCTAATACGACTCACTATAGGCGCAGTGGGCAGCTGTT
dsGFP(T7)-F		GGATCCTAATACGACTCACTATAGGGTAAACGGCCAC AAGTTCAG
dsGFP(T7)-R		GGATCCTAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTC
e-PAP-F	PAP qRT-PCR	CTGTCCACGCCAAGATTC
e-PAP-R		CACCGCTTTCTCCAGTC