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Purification and Characterization of a Small Cationic Protein from the Tobacco Hornworm *Manduca sexta*

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Abstract

The prophenoloxidase (proPO) activation system is an important defense mechanism in arthropods, and activation of proPO to active phenoloxidase (PO) involves a serine proteinase cascade. Here, we report the purification and characterization of a small cationic protein CP8 from the tobacco hornworm, *Manduca sexta*, which can stimulate proPO activation. BLAST search showed that *Manduca* CP8 is similar to a fungal proteinase inhibitor-1 (AmFPI-1), an inducible serine proteinase inhibitor-1 (ISPI-1), and other small cationic proteins with unknown functions. However, we showed that *Manduca* CP8 did not inhibit proteinase activity, but stimulated proPO activation in plasma. When small amount (0.1 µg) of purified native CP8 or BSA was added to cell-free plasma samples and incubated for 20 min, low PO activity was observed in both groups. But significantly higher PO activity was observed in the CP8-group than in the BSA-group when more proteins (0.5 µg) were added and incubated for 20 min. However, when the plasma samples were incubated with proteins for 30 min, high PO activity was observed in both the CP8 and BSA groups regardless the amount of proteins added. Moreover, when PO in the plasma was pre-activated with *Micrococcus luteus*, addition of CP8 did not have an effect on PO activity, and CP8/bacteria mixture did not stimulate PO activity to a higher level than did BSA/bacteria. These results suggest that CP8 helps activate proPO more rapidly at the initial stage. CP8 mRNA was specifically expressed in fat body and its mRNA level decreased when larvae were injected with saline or bacteria. However, CP8 protein concentration in hemolymph did not change significantly in larvae injected with saline or microorganisms.

Keywords

prophenoloxidase; phenoloxidase; serine proteinase; cationic protein; innate immunity; *Manduca sexta*.

1. Introduction

The prophenoloxidase (proPO) activation system is an important component of the immune system in arthropods (Cerenius et al., 2008; Cerenius and Soderhall, 2004). For example, in the freshwater crayfish, *Pacifastacus leniusculus*, depletion of proPO by RNA interference leads to lower phenoloxidase (PO) activity, but increased bacterial growth and higher mortality

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when infected with a highly pathogenic bacterium, *Aeromonas hydrophila* (Liu et al., 2007a). In the wasp *Microplitis demolitor*, suppression of melanization response by a novel inhibitor from the *M. demolitor* bracovirus is functionally important for both the virus and the wasp (Beck and Strand, 2007). In mosquitoes, melanization response contributes to the killing of the rodent parasite *Plasmodium berghei* in *Anopheles gambiae* (Abraham et al., 2005; Michel et al., 2005; Osta et al., 2004; Volz et al., 2006), and *P. yoelii* is melanized in the naturally refractory strain *An. dirus* (Wen-Yue et al., 2007). In addition, antimalarial drugs can induce melanotic encapsulation of *Plasmodium* by *An. stephensi* (Zhang et al., 2008). PO activity also has an effect on bacterial growth, for example, *Escherichia coli* and *Bacillus subtilis* cease to grow when treated with *Manduca sexta* PO and dopamine *in vitro* (Zhao et al., 2007). However, it has been shown that increasing melanizing activity in *An. gambiae* does not affect development of the human malaria parasite *P. falciparum* (Michel et al., 2006). ProPO activation and melanization are also not essential for defense against bacteria in *An. gambiae* and *Drosophila melanogaster* (Leclerc et al., 2006; Schnitger et al., 2007), this may be because cellular immunity such as phagocytosis is central to the host defense against bacteria in mosquitoes and *Drosophila* (Matova and Anderson, 2006).

Activation of the zymogen proPO to the active PO involves a serine proteinase cascade (Cerenius et al., 2008; Cerenius and Soderhall, 2004). ProPO is cleaved by proPO-activating proteinases (PAPs), which are also present as pro-enzymes in hemolymph (Gorman et al., 2007; Jiang et al., 1998; Jiang et al., 2003a; Jiang et al., 2003b; Wang and Jiang, 2007). Therefore, proPO activation is tightly regulated by serine proteinases and serpins (serine proteinase inhibitors) (Tong et al., 2005; Tong and Kanost, 2005; Zhu et al., 2003). ProPO activation can also be positively or negatively modulated by non-enzymatic proteins such as serine proteinase homologs (SPHs) (Asgari et al., 2003; Volz et al., 2006; Yu et al., 2003; Zhang et al., 2004), PO inhibitors (Lu and Jiang, 2007), and lectins (Yu et al., 1999; Yu and Kanost, 2000).

In this study, we purified a small cationic protein from hemolymph of the tobacco hornworm, *M. sexta*, and cloned its cDNA. BLAST search showed that the deduced amino acid sequence of this *Manduca* protein (named cationic protein CP8) is similar to a fungal proteinase inhibitor-1 (AmFPI-1) of the Indian tasar silkworm *Antheraea mylitta* (Shrivastava and Ghosh, 2003) and an inducible serine proteinase inhibitor-1 (ISPI-1) of the wax moth *Galleria mellonella* (Fröblius et al., 2000), as well as to a small cationic protein from *Bombyx mori* (accession number: **AY655143**) and *B. mandarina* (accession number: **EF126182**), and an unknown protein from *Picea sitchensis* (accession number: **ABK25652**). Both AmFPI-1 and ISPI-1 have inhibitory activity against some fungal serine proteinases and trypsin. However, we found that the purified *Manduca* CP8 did not inhibit the activity of seven serine proteinases tested, but stimulated proPO activation in plasma. When small amount (0.1 µg) of purified native CP8 or BSA was added to cell-free plasma samples and incubated for 20 min, low PO activity was observed in both groups. But significantly higher PO activity was observed in the CP8-group than in the BSA-group when more proteins (0.5 µg) were added and incubated for 20 min. However, when the plasma samples were incubated with proteins for 30 min, high PO activity was observed in both the CP8 and BSA groups regardless the amount of proteins added. Moreover, when PO in the plasma was pre-activated with *Micrococcus luteus*, addition of CP8 did not have an effect on PO activity, and CP8/bacteria mixture did not stimulate PO activity to a higher level than did BSA/bacteria. These results suggest that CP8 helps activate proPO more rapidly at the initial stage. CP8 mRNA was specifically expressed in fat body and its mRNA level decreased in larvae injected with saline or bacteria. However, CP8 protein concentration in hemolymph did not change in *M. sexta* larvae injected with saline or microorganisms. In addition, CP8 interacted with immulectin-3 (IML-3), a C-type lectin that can enhance cellular encapsulation (Yu et al., 2005).

2. Materials and Methods

2.1. Insects

M. sexta eggs were kindly provided by Dr. Michael Kanost, Department of Biochemistry, Kansas State University. Larvae were reared on an artificial diet at 25°C (Dunn and Drake, 1983). The fifth instar larvae were used for the experiments.

2.2. Purification of cationic protein CP8 from hemolymph of *M. sexta* larvae

Day 2 fifth instar *M. sexta* naïve larvae were injected with lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4) (20 µg per larva) and hemolymph was collected 24h post-injection. Cell-free plasma was collected by centrifugation at 3,300 g for 5 min at 4°C and stored at -80°C for protein purification. CP8 was purified as one of the plasma proteins that can interact with recombinant immulectin-3 (IML-3) (Yu et al., 2005). Several steps of chromatography were applied to purify these plasma proteins as outlined in Fig. 1B. Far western blot analysis was used to detect the desired proteins in fractions from each purification step.

Plasma proteins were first precipitated with saturated ammonium sulfate to a final of 50% and centrifuged at 10,000 g for 15 min at 4°C. The desired proteins were in the supernatant, which was dialyzed against 20 mM Tris-HCl (pH 6.8) for 24h at 4°C with one buffer change. Then the dialyzed sample was loaded to a Macro-Prep High-Q column (Bio-Rad) (~45 ml) pre-equilibrated with 20 mM Tris-HCl (pH 6.8) at 1 ml/min. The desired proteins did not bind to the High-Q column and were in the flow-through. The High-Q column flow-through was dialyzed against 20 mM Na₂HPO₄ (pH 6.8) for 24h at 4°C with one buffer change, and loaded to a Macro-Prep High-S column (Bio-Rad) (~45 ml) pre-equilibrated with 20 mM Na₂HPO₄ (pH 6.8) at 0.5 ml/min. Then the High-S column was washed with 20 mM Na₂HPO₄ (pH 6.8) until the OD₂₈₀ was less than 0.001, and eluted with an increasing salt gradient (0.01–1M NaCl) in 20 mM Na₂HPO₄ (pH 6.8) (total 90 ml). Fractions of 1.5 ml were collected. Fractions containing the desired proteins from the High-S column were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.2) for 24h at 4°C with one buffer change, and then loaded to a pre-packed Econo-Pac® CHT-II Cartridge (Bio-Rad) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.2) at 0.25 ml/min. The column was washed with 10 mM sodium phosphate buffer (pH 7.2) until OD₂₈₀ was less than 0.001, and then eluted with an increasing gradient of phosphate (pH 7.2) (10–500 mM) (total 30 ml) at 0.25 ml/min. Fractions of 0.8 ml were collected. Two proteins with apparent molecular weights of ~ 10-kDa and 14-kDa were co-purified.

Finally, a gel filtration column was performed to further separate the two proteins. The dry medium of Bio-Gel P-100 Gel (Bio-Rad) was hydrated in 20 mM phosphate, 30 mM NaCl (pH 6.8) and carefully packed to a column (2.5×120 cm, Bio-Rad) up to 90% of the column length (~530 ml packed gels). The column was washed and equilibrated with the same buffer. Fractions containing the 10-kDa and 14-kDa proteins were pooled and concentrated to 5 ml and then loaded to the column at one time. The phosphate buffer was pumped at 0.1 ml/min. The first 100 ml fractions were collected together. Then fractions of 5 ml were collected. Fractions containing the purified 10-kDa or 14-kDa protein were pooled separately and concentrated. Finally, the concentrated proteins were dialyzed against a Tris buffer (20 mM Tris-HCl, 10 mM NaCl, pH 6.8) and centrifuged at 10,000 g for 15 min at 4°C to remove any precipitates. The protein concentration was determined by the Bradford method (Bradford, 1976), and the 10-kDa protein was named cationic protein CP8.

2.3. Determination of the amino-terminal sequence and molecular mass of the purified CP8

The purified native CP8 was separated on a Tris-Tricine gel and transferred to a nitrocellulose membrane. The membrane was stained in 0.5% amido black for 1–2 min, de-stained in 1%

acetic acid, and washed in water several times. The protein band was then cut out and sent to the Biotech Core Facility at Kansas State University for determination of the amino-terminal sequence. The mass of CP8 was also determined by MALDI-TOF at the Biological Mass Spectrometry and Proteomics Facility, University of Missouri – Kansas City. Insulin was used as an internal molecular weight standard.

2.4. Cloning of the full-length CP8 cDNA

Total RNA was prepared from fat body of the day 2 fifth instar *M. sexta* naïve larvae using the Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Both the 5'- and 3'-RACE (rapid amplification of cDNA ends) were performed to clone the full length CP8 cDNA. The cDNA for the 3'-RACE was synthesized from the total RNA of fat body (1.65 µg) using the ThermoScript™ RT-PCR System (Invitrogen) and the Adaptor-dT₁₇ primer (5'-GGC CAC GGG TCG ACT AGT ACT (T)₁₇- 3'), and the cDNA for the 5'-RACE was synthesized from the total RNA of fat body using the PowerScript™ Reverse Transcriptase (Clontech) and Oligo (dT)₂₀ primer and 5'-CDS primer (5'- AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ATG GCC GGG -3'). All cDNAs were stored at -20°C.

A degenerate primer K216F (5'- GTN TGY GGN WSN AAY TAY TAY TG- 3') was designed based on the amino-terminal sequence (VCGSNYYC) of CP8, and the 3'-RACE was performed using the Easy-A® High-Fidelity PCR Cloning Enzyme (Stratagene), K216F and Adaptor-dT₁₇ primers, and 1 µl of the 3'-RACE cDNA as a template. PCR reactions were performed as followings: initial denaturing at 94°C for 2 min, then 35 cycles of denaturing at 94°C for 30s, annealing at 55°C for 45s, and extension at 72°C for 60s, followed by a final extension at 72°C for 10 min. A PCR product of ~400 bp was purified, cloned into plasmid vector pGEMR-T (Promega) and sequenced.

Based on the nucleotide sequence of the 3' cDNA fragment, a gene-specific reverse primer K219R (5'-GAG GGT CAC GAC AGA TGG CGG ATG- 3') was designed for the 5'-RACE. PCR reaction was performed the same as described for the 3'-RACE using the 5'-RACE cDNA as a template and nested universal primer (NUP) (5'-AAG CAG TGG TAT CAA CGC AGA GT- 3') and K219R primers. The PCR product was purified, cloned and sequenced. The full length cDNA of CP8 was then assembled by overlapping the 3' and 5' cDNA fragments.

2.5. Expression of recombinant CP8 and production of rabbit polyclonal antibody

The cDNA fragment encoding amino acids 20–105 of CP8 (mature protein, Fig. 2A) was generated by PCR using CP8-F (5'-GCC ATG GTG TGC GGC TCA AAC TAC TGC- 3') and CP8-R (5'-TTA AAG CTT AGG AGC GAC CCT GGT AC- 3') primers. The cDNA fragment was cloned into plasmid vector pGEMR-T and the insert was excised by *Nco* I and *Hind* III. The digested fragment was then separated, recovered and cloned into the *Nco* I/*Hind* III sites of the expression vector H6pQE-60 (Lee et al., 1994). Recombinant CP8 was expressed in *E. coli* strain XL1-Blue (Stratagene) as an inclusion body and purified under denaturing conditions in 8M urea using nickel-nitrilotriacetic acid (Ni-NTA) resins (Qiagen) and re-natured by a three-step dialysis as described previously (Yu et al., 2005). Purified recombinant CP8 in 8M urea was further separated on a preparative SDS-PAGE, and the gel slice containing recombinant CP8 (0.6 mg) was cut out and used as an antigen for the production of polyclonal rabbit antiserum (Cocalico Biologicals, Inc.).

2.6. SDS-PAGE, Western blot and Far western blot analyses

Plasma samples, protein fractions, or purified proteins were separated on 15% SDS-PAGE by the method of Laemmli (1970), and the gel was visualized by Coomassie blue staining. For Western blot analysis, proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% dry skim milk in Tris-buffered saline containing Tween-20 (TBS-T)

(25mM Tris-HCl, 137mM NaCl and 3mM KCl, pH 8.0, 0.05% Tween-20) and then incubated with polyclonal rabbit antibody to recombinant CP8 (1:2000). Antibody binding was visualized by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (1:3000) (Bio-Rad). Far western blot was performed according to the method of Einarson and Orlinick (2002). Proteins were separated on 15% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was washed in TBS-T for 1h with a buffer change every 20 min to remove SDS. Then the membrane was probed with recombinant IML-3 (rIML-3) or CP36 (rCP36), a cuticle protein from *M. sexta* (Suderman et al., 2003) as a control (each at 1 µg/ml) in TBS-T containing 0.1% BSA for 2h at room temperature. A control membrane was also probed in TBS-T containing 0.1% BSA without rIML-3 or rCP36. Binding of rIML-3 to proteins was detected by mouse monoclonal antibody to the His-tag (1:5000), followed by goat anti-mouse IgG conjugated to alkaline phosphatase (1:5000) as described for western blot analysis above.

2.7. Tissue distribution of CP8

Hemocytes, fat body, epidermis, midgut and Malpighian tubule were collected or dissected from four *M. sexta* fifth instar naïve larvae (day 2), and total RNAs were prepared from these tissues using the Trizol Reagent (Invitrogen). Residual DNA in the RNA samples was removed by DNase (RQ1). RNA samples were stored at -80°C.

To determine tissue distribution of CP8, real-time PCR was performed using 2×SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen). cDNAs were prepared from RNA samples of different tissues from naïve larvae as described above using the Thermoscript RT-PCR system. Real-time PCR reactions were carried out in 25-µl reactions. For amplification of ribosomal protein S3 (RPS3) gene (Jiang et al., 1996), K12 (5'-TTA ATT CCG AGC ACT CCT TG- 3') and K13 (5'-GGA GCT GTA CGC TGA GAA AG- 3') primers and 0.5 µl of 1:10 diluted cDNA were used. For CP8 gene, CP8-F and CP8-R primers and 1 µl of 1:10 diluted cDNA were used. Amplification was performed by incubation at 50°C for 2min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1min. PCR products were detected by agarose gel analysis after each PCR reaction to confirm the specific gene amplification. Data from three repeats of each sample were analyzed by the ABI 7500 SDS software (Applied Biosystem, USA) using a comparative method ($2^{-\Delta\Delta C_t}$) as described previously (Ao et al., 2008). All the data were presented as relative mRNA expression (means of three individual measurements ± SEM).

2.8. Expression of CP8 mRNA in fat body and CP8 protein in hemolymph of *M. sexta* larvae after immune-challenge

Day 2 fifth instar *M. sexta* naïve larvae were injected with saline, formaldehyde-killed *E. coli* XL1-Blue (10^8 cells per larva), *M. luteus* (100 µg per larva), or yeast (*Saccharomyces cerevisiae*) (10^7 cells per larva) (at least four larvae in each group). Hemolymph samples were collected at 0 and 24 h post-injection for protein analysis, whereas fat body was collected at 24 h post-injection for RNA preparation. To determine CP8 mRNA expression in fat body after immune-challenge, real-time PCR was performed the same as described above. To detect CP8 protein in hemolymph, plasma samples from 4 larvae were equally mixed and analyzed by SDS-PAGE, and CP8 protein was identified by immunoblotting using polyclonal rabbit antibody to recombinant CP8.

2.9. Serine proteinase inhibition assay

Proteinase inhibition assay was performed with purified native and recombinant CP8 using the method described for ISPI-1 (Fröblius et al., 2000). Ten microliters of native or recombinant CP8 (each at 0.5 µg) or BSA (0.5 µg) was pre-incubated with 10µl of a proteinase (all were prepared in the assay buffer, 100 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, pH 7.5) for 30

min at room temperature. Then a specific peptide substrate (200 μ l, 50 μ M) prepared in the same assay buffer was added, and the yellow p-nitroanilide produced from the digested peptide substrate was monitored by measuring absorbance at 405 nm every min for 30 min. Substrates alone were used as blanks. One unit of enzyme activity is defined as an increase of absorbance (ΔA_{405}) by 0.001 per minute. We tested different amounts of proteinases in order to use suitable concentrations of proteinases for the assay. The serine proteinases and peptide substrates used in our assays were all purchased from Sigma: bovine pancreatic trypsin (5 ng/ μ l) and N-benzoyl-Val-Gly-Arg-p-nitroanilide, bovine pancreatic chymotrypsin (10 ng/ μ l) and N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, porcine pancreatic elastase (20 ng/ μ l) and N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide, subtilisin A (20 ng/ μ l) and N-benzoyl-Phe-Val-Arg-p-nitroanilide, proteinase K (20 ng/ μ l) and N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide, and a proteinase from *Streptomyces griseus* (20 ng/ μ l) and N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. We also tested the effect of CP8 on proPO-activating proteinase-3 (PAP-3) (Jiang et al., 2003b). Active PAP-3 fraction and the substrate N-succinyl-Ile-Glu-Ala-Arg-p-nitroanilide were kindly provided by Dr. Michael Kanost, Kansas State University. PAP-3 (~10ng each) was pre-incubated with purified native or recombinant CP8, or with BSA (each at 0.5 μ g) in 20 μ l of the assay buffer for 30 min at room temperature, then the N-succinyl-Ile-Glu-Ala-Arg-p-nitroanilide substrate (200 μ l, 50 μ M) was added and the PAP-3 activity was measured as described above.

2.10. Prophenoloxidase activation assay

We first screened naïve plasma samples for proPO activation assay. Aliquots of plasma samples (2 μ l each) from individual naïve larvae were incubated with or without *M. luteus* (0.5 μ g each) in 10 μ l of TBS (pH 7.4) in wells of a 96-well plate for 60 min at room temperature. Then L-dopamine (2 mM in 50 mM sodium phosphate buffer, pH 6.5) was added (200 μ l per well) and absorbance at 470nm was monitored with time in a microtiter plate reader (PowerWave XS, Bio-Tek Instrument, Inc.). One unit of PO activity is defined as an increase of absorbance (ΔA_{470}) by 0.001 per minute. Plasma samples with low or no PO activity when incubated alone (without *M. luteus*) but high PO activity after incubation with *M. luteus* are selected for proPO activation assays.

For proPO activation assays, naïve plasma #31 (2 μ l each) was incubated with heat-treated (65°C, 30 min) purified native CP8 or BSA (0.1 or 0.5 μ g each) in a total of 10 μ l TBS (pH 7.4) for 20 or 30 min. Naïve plasma sample #31 (2 μ l each) was also incubated with buffer alone (Control), CP8/*M. luteus* mixture (0.5 μ g each) or BSA/*M. luteus* mixture (0.5 μ g each) for 10 min at room temperature, or pre-activated with *M. luteus* (0.5 μ g each) for 5 min at room temperature, and then incubated with native CP8 (0.5 μ g) or BSA (0.5 μ g) for another 5 min (totally 10 min). Finally, L-Dopamine substrate (200 μ l, 2 mM) was added to each sample, and PO activity was measured at 470 nm in a plate reader for 30 min. Data from four replicas of each sample were analyzed. These experiments were repeated at least 3 times and similar results were obtained. One representative set of data was used to make figures using the GraphPad Prism software.

2.11. Sequence Analysis

Multiple sequence alignment was performed by a ClustalW program (<http://www.ch.embnet.org/software/ClustalW.html>). Molecular weight and isoelectric point (pI) of CP8 were calculated using programs at <http://www.ualberta.ca/~stothard/javascript>. The significance of difference was determined by an unpaired t-test using the GraphPad InStat software (GraphPad, San Diego, CA).

3. Results

3.1. Purification of cationic protein CP8

In our attempts to identify hemolymph proteins that can interact with immulectin-3 (IML-3) using a protein pull-down assay, we found that some plasma proteins with low molecular weights from *M. sexta* larvae were pulled down by recombinant IML-3 (rIML-3) (data not shown). We then performed a far western blot analysis to directly detect protein-protein interactions between rIML-3 and *M. sexta* larval plasma proteins (Fig. 1A). Two plasma proteins with apparent molecular weights of about 10- and 14-kDa specifically interacted with rIML-3 (Fig. 1A, lane 2), but no plasma proteins interacted with recombinant CP36 (rCP36), a cuticle protein from *M. sexta* larvae (Suderman et al., 2003) (data not shown). Also, no plasma proteins interacted with primary and secondary antibodies alone when rIML-3 was omitted (data not shown).

To purify these two plasma proteins, ammonium sulfate precipitation combined with several steps of chromatography were applied (Fig. 1B). For each purification step, far western blot was used to detect the two proteins. These two proteins were in the supernatant of 50% ammonium sulfate and did not bind to the anion exchange column. After cation exchange and HT (hydroxylapatite) columns, these two proteins were co-purified. The two proteins were finally separated by a gel filtration chromatography. The 10-kDa protein was purified to homogeneity and appeared as a single protein band in SDS-PAGE (Fig. 1C, lane 2). We named the 10-kDa protein as cationic protein CP8. The 14-kDa protein has been identified as *M. sexta* lysozyme (E Ling and XQ Yu, unpublished results). The purified CP8 has a molecular weight of 9094.32 Da determined by mass spectrometry (Fig. 1D). No mass signal around 14-kDa corresponding to *M. sexta* lysozyme (with a calculated monoisotopic mass of 13974.74 Da) was observed in the mass spectrum (data not shown), indicating that the purified CP8 was not contaminated with lysozyme.

3.2. Cloning of the full-length CP8 cDNA

The amino-terminal sequence of CP8 was determined by Edman degradation and fifteen amino acid residues were obtained (Fig. 2A). BLAST search showed that the amino-terminal sequence (15 residues) of CP8 is similar to that of a fungal proteinase inhibitor-1 (AmFPI-1) of *A. mylitta* (Shrivastava and Ghosh, 2003), a cationic peptide CP8 from *Bombyx mori* (accession number: [AAU08172](#)) and *B. mandarina* (accession number: [ABL76064](#)), and an unknown protein from *Picea sitchensis* (accession number: [ABK25652](#)).

To clone the full-length cDNA of CP8, a degenerate primer based on the amino-terminal sequence of CP8 was designed for the 3'-RACE and a fragment of 400 bp was obtained. Then a gene-specific primer was designed for the 5'-RACE, and the full length cDNA was obtained by assembling the two overlapping fragments. The full length cDNA of CP8 is 501 bp (accession number: [EF467286](#)), with an open reading frame (ORF) of 318 bp that encodes a polypeptide of 105 residues (Fig. 2A). CP8 contains a secretion signal peptide of 19 residues, and the molecular weight of the mature CP8 calculated from the deduced amino acid sequence is 9.11 kDa, which matches the mass (9094.32 Da) determined by mass spectrometry (Fig. 1D). CP8 has a predicted isoelectric point (pI) of 8.43. It contains 12 cysteine residues, but does not have N- or O-linked glycosylation sites. *M. sexta* CP8 is most similar to the AmFPI-1 (73% identity) from *A. mylitta* (Shrivastava and Ghosh, 2003), followed by an unknown protein from *P. sitchensis* (67%), the cationic peptide CP8 from *B. mori* (61%) and *B. mandarina* (59%), and the inducible serine proteinase inhibitor-1 (ISPI-1) (50%) from *G. mellonella* (Fröbius et al., 2000) (Fig. 2B). This group of proteins all contains 12 cysteines, which may form 6 disulfide bonds. AmFPI-1 and ISPI-1 have inhibitory activity against some fungal serine proteinases and trypsin *in vitro* (Shrivastava and Ghosh, 2003; Fröbius et al., 2000).

3.3. CP8 mRNA is specifically expressed in fat body and the protein is secreted into hemolymph

We had expressed recombinant mature CP8 (residues 20–105, Fig. 2A) in *E. coli* and purified the recombinant protein to homogeneity by nickel affinity chromatography as indicated by a single protein band on SDS-PAGE (Fig. 1C, lane 1). The purified recombinant CP8 was used as an antigen to inject rabbits to produce polyclonal antiserum. Recombinant CP8 was also refolded by a 3-step dialysis as described previously (Yu et al., 2005).

To determine expression of CP8 mRNA in tissues, real-time PCR was performed (Fig. 3A). CP8 mRNA was specifically expressed in fat body, but not in other tissues such as hemocytes, midgut, Malpighian tubules or epidermis of naïve larvae. CP8 transcript in fat body was significantly down-regulated after larvae were injected with saline or bacteria (*E. coli* or *M. luteus*) (Fig. 3B). Expression of CP8 mRNA in fat body was also decreased but not significantly in larvae injected with yeast. However, the concentration of CP8 protein in hemolymph did not change significantly when larvae were injected with saline or microorganisms (Fig. 3C). CP8 protein was not detected in hemocytes of naïve or immune-challenged larvae (data not shown).

3.4. CP8 does not have an effect on serine proteinase activity

CP8 is highly similar to AmFPI-1 and ISPI-1 (Fig. 2B). AmFPI-1 is an inhibitor of a fungal proteinase from *Aspergillus oryzae* and it also inhibits bovine trypsin and chymotrypsin (Shrivastava and Ghosh, 2003). ISPI-1 inhibits trypsin and trypsin-like proteinase PR2 from the entomopathogenic fungus *Metarhizium anisopliae*, but increases the activity of chymotrypsin and proteinase K by 25% and 50%, respectively (Fröblius et al., 2000).

To test whether CP8 has an effect on serine proteinases, we performed a proteinase assay using a method similar to the one described for ISPI-1 (Fröblius et al., 2000). We tested seven serine proteinases, including trypsin, chymotrypsin, elastase, subtilisin A, proteinase K, a proteinase from the bacterium *Streptomyces griseus*, and proPO-activating proteinase-3 (PAP-3) that can activate *M. sexta* proPO (Jiang et al., 2003b), at different concentrations, however, both native and recombinant CP8 did not inhibit or increase the activity of the seven serine proteinases tested (Fig. 4).

3.5. CP8 stimulates prophenoloxidase activation in plasma

CP8 interacted with immulectin-3 (IML-3) (Fig. 1A), a C-type lectin from *M. sexta*. We have demonstrated that immulectins have functions in proPO activation, encapsulation and melanization (Yu and Kanost, 2000;2004;Yu et al., 1999;2005;2006;Ling and Yu, 2006). To test whether CP8 plays a role in proPO activation, cell free plasma was collected from naïve larvae and incubated with purified native CP8 or BSA (as a control protein). ProPO activation was then monitored and PO activity was measured. We used naïve plasma #31 that contains ~142 µg/mL CP8 (Fig. 5D and 5E) for all the proPO activation assays. When small amount (0.1 µg to 2 µl plasma) of heat-treated (65°C, 30 min) native CP8 or BSA was added to the plasma samples (#31) and incubated for 20 min, low PO activity was observed in both groups and the difference in PO activity between the CP8-group (5.3 units) and the BSA-group (4.5 units) was not significant ($p=0.0672$) (Fig. 5A). However, when more proteins (0.5 µg to 2 µl plasma) were added to the plasma samples and incubated for 20 min, significantly ($p=0.0012$) higher PO activity was observed in the CP8-group (13.7 units) than in the BSA-group (7.4 units) (Fig. 5A). When the plasma samples were incubated with proteins for a longer period of time (30 min), high PO activity was observed in both the CP8 and BSA groups regardless the amount of proteins added (56 and 69 units for 0.1 and 0.5 µg BSA, 56 and 61 units for 0.1 and 0.5 µg CP8, respectively) (Fig. 5B). We also tested the effect of CP8/*M. luteus* or BSA/*M. luteus* mixture on proPO activation and found that high PO activity was observed in both

groups and there was no significant difference in PO activity between the two groups ($p=0.4523$) (Fig. 5C). Moreover, when PO in the plasma was pre-activated with *M. luteus*, addition of CP8 did not have an effect on PO activity (Fig. 5C). These results suggest that CP8 helps activate proPO more rapidly in plasma but it does not increase PO activity after proPO is activated.

4. Discussion

We have purified a small cationic protein CP8 from the plasma of *M. sexta* larvae, which is similar to AmFPI-1 of *A. mylitta* (Shrivastava and Ghosh, 2003), ISPI-1 of *G. mellonella* (Fröbisch et al., 2000), a cationic protein CP8 of *B. mori* and *B. mandarina*, and an unknown protein of *P. sitchensis* (Fig. 2B). These proteins are cationic and small in size (~9 kDa for mature proteins) but rich in disulfide bonds (12 cysteines). Both AmFPI-1 and ISPI-1 have inhibitory activity against some fungal proteinases and trypsin, but functions of *B. mori*, *B. mandarina* and *P. sitchensis* proteins have not been characterized. However, this family of proteins does not show sequence similarity to the Kazal family of proteinase inhibitors that contain three disulfide bonds (Rawlings et al., 2004).

We showed that *Manduca* CP8 did not inhibit or increase the activity of seven serine proteinases, including proPO-activating proteinase-3 (PAP-3) (Fig. 4), but stimulated proPO activation in plasma (Fig. 5). CP8 concentration in plasma varies between 150–250 $\mu\text{g/mL}$ (Figs. 3C, 5D and 5E). The naïve plasma #31 used in the proPO activation assays contains ~142 $\mu\text{g/mL}$ CP8 (Fig. 5D and 5E). With short incubation time (20 min), addition of higher concentration of CP8 (0.5 μg to 2 μL plasma, 250 $\mu\text{g/mL}$ final concentration, ~1.8-fold of the endogenous CP8) activated PO to a significantly higher activity compared to the control BSA (0.5 μg) (Fig. 5A), whereas small amount of CP8 (0.1 μg to 2 μL plasma, 50 $\mu\text{g/mL}$ final concentration) and BSA only increased PO activity to a similarly low level (Fig. 5A), indicating that CP8 can stimulate proPO activation in a concentration-dependent manner. When the incubation time was increased to 30 min, high PO activity was observed in both the CP8 and BSA groups despite the amount of proteins added (Fig. 5B). These results suggest that CP8 at certain high concentrations can stimulate proPO activation more rapidly at the initial stage but can not increase PO activity after proPO is already activated. At 20 min incubation time, most proPO in the plasma was not converted to PO, thus addition of CP8 (0.5 μg) stimulated the activation process. However, at 30 min incubation time, proPO was already converted to PO in the plasma alone, and because the proPO activation is a rapid process, so addition of CP8 did not increase PO activity. This conclusion is further supported by the observations that CP8/*M. luteus* and BSA/*M. luteus* mixtures both activated PO to a high activity (72 and 66 units, respectively) (Fig. 5C), and CP8 did not have an effect on PO activity when PO was pre-activated by *M. luteus* in the plasma (Fig. 5C). When protein/*M. luteus* was added to the plasma, bacteria can activate proPO more rapidly and easily (via activation of the serine proteinase cascade), so the effect of CP8 could not be observed. CP8 may play a role in innate immunity by promoting melanization of microbes since it can interact with IML-3, a C-type lectin pattern recognition receptor (PRR) that binds microbes (Yu et al., 2005). When IML-3 binds to microbial surface, it may recruit CP8 to the surface of microbes and elevate local concentration of CP8, thus CP8 can stimulate proPO activation on the microbial surface and promote melanization of microbes. However, the proPO activation system is a very complicated system, which involves serine proteinases (Gorman et al., 2007; Jiang et al., 1998; Jiang et al., 2003a; Jiang et al., 2003b; Wang and Jiang, 2007), proteinase inhibitors (Tong et al., 2005; Tong and Kanost, 2005; Zhu et al., 2003), and other modulating proteins such as immunolectins and serine proteinase homologs (Yu et al., 1999; 2003; Yu and Kanost, 2000). Thus, CP8 may be one of the modulating proteins that can stimulate the proPO activation process. We did not detect direct interaction of CP8 with proPO/PO by far western blot analysis, and incubation of

CP8 with plasma did not have an effect on PO activity after activation with CPC (cetylpyridinium chloride) (data not shown).

Manduca CP8 is highly similar (73% identity) to AmFPI-1 of *A. mylitta* (Fig. 2B), but CP8 did not have inhibitory activity against seven serine proteinases tested. We do not know whether CP8 has inhibitory activity against fungal proteinases or not, since we can not obtain fungal proteinase samples. Considering insect immune responses to fungal infection, CP8, AmFPI-1 and ISPI-1 may have similar functions. AmFPI-1 and ISPI-1 have direct inhibitory activity against fungal proteinases that may increase pathogenicity by degrading insect cuticles (Donatti et al., 2008) to limit fungal infection in insects. CP8 stimulates proPO activation, an important immune response against invading microorganisms including fungi (Mullen and Goldsworthy, 2006; Schwarzenbach and Ward, 2007). We speculate that this family of proteins may interact with proteinases to modulate (either enhance or inhibit) their activity. But CP8 did not have an effect on PAP-3 activity (Fig. 4). It is possible that CP8 requires other proteins for interacting with either proPO-activating proteinases (PAPs) or their upstream proteinases in the proPO activation system. Thus, CP8 in the plasma interacts with proteinases to stimulate proPO activation, while AmFPI-1 and ISPI-1 interact with fungal proteinases to inhibit their activity (Fröblius et al., 2000; Shrivastava and Ghosh, 2003). We also notice that ISPI-1 increases the activity of chymotrypsin and proteinase K by 25% and 50%, respectively, but does not have an effect on the activity of papain or subtilisin (Fröblius et al., 2000). Thus, this family of small cationic proteins may modulate proteinase activity by protein-protein interactions. Such protein-enzyme interactions may allosterically affect the proteinase activity. CP8 resisted digestion by serine proteinases even after a long period of incubation (12h), and no CP8-proteinase complex was observed in the *M. sexta* larval plasma (data not shown). Thus, the mechanism used by CP8, AmFPI-1 and ISPI-1 to enhance or inhibit proteinase activity differs from the one used by serpins (serine proteinases inhibitors), which inhibit serine proteinase activity by formation of a covalent bond with proteinases to block the substrate binding (Liu et al., 2007b; Swanson et al., 2007). However, it is not clear how interaction of these small cationic proteins with different proteinases can either enhance or inhibit the enzymatic activity. One possibility is that these proteins may have a similar core structure but different surfaces (charges and/or hydrophobicity) for protein-protein interactions with proteinases. Interaction of a small cationic protein with serine proteinases may cause conformational changes in the proteinase, which either enhance or inhibit the activity. Whether a proteinase activity is enhanced or inhibited by a cationic protein depends upon both the properties of the cationic protein and the enzyme.

CP8 was specifically expressed in fat body and secreted into hemolymph. Real-time PCR showed that CP8 mRNA was significantly decreased in fat body after larvae were injected with saline (injury) or bacteria (infection). CP8 transcript was also decreased but not significantly after injection with yeast (Fig. 3B). However, CP8 protein level in plasma was not affected by injury or microbial infections (Fig. 3C). One reason for the small changes of CP8 protein in hemolymph may be that CP8 is not degraded by its associated proteinases. Another possibility is that the level of serine proteinases in hemolymph that require CP8 is not high and only very little CP8 is used. Future work is to investigate how CP8 stimulates proPO activation.

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The abbreviations used are

AmFPI-1

	<i>A. mylitta</i> fungal proteinase inhibitor-1
CP8	cationic protein
IML-3	immulectin-3
rIML-3	recombinant IML-3
ISPI-1	inducible serine proteinase inhibitor-1
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
proPO	prophenoloxidase
PO	phenoloxidase
PAP	proPO-activating proteinase
RACE	rapid amplification of cDNA ends
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TBS-T	TBS containing 0.005% Tween-20

References

- Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M, Michel K. An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc Natl Acad Sci USA* 2005;102:16327–16332. [PubMed: 16260729]
- Ao JQ, Ling E, Yu XQ. A Toll receptor from *Manduca sexta* is in response to *Escherichia coli* infection. *Mol Immunol* 2008;45:543–552. [PubMed: 17606296]
- Asgari S, Zhang G, Zareie R, Schmidt O. A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits melanization of the host hemolymph. *Insect Biochem Mol Biol* 2003;33:1017–1024. [PubMed: 14505695]
- Beck MH, Strand MR. A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway. *Proc Natl Acad Sci USA* 2007;104:19267–19272. [PubMed: 18032603]
- Bradford M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem* 1976;72:248–254. [PubMed: 942051]
- Cerenius L, Lee BL, Soderhall K. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol* 2008;29:263–271. [PubMed: 18457993]

- Cerenius L, Soderhall K. The prophenoloxidase-activating system in invertebrates. *Immunol Rev* 2004;198:116–126. [PubMed: 15199959]
- Donatti AC, Furlaneto-Maia L, Fungaro MH, Furlaneto MC. Production and regulation of cuticle-degrading proteases from *Beauveria bassiana* in the presence of *Rhammatocerus schistocercoides* cuticle. *Curr Microbiol* 2008;56:256–260. [PubMed: 18071791]
- Dunn PE, Drake D. Fate of bacteria injected into naive and immunized larvae of the tobacco hornworm, *Manduca sexta*. *J Invertebr Pathol* 1983;41:77–85.
- Einarson, MB.; Orlinick, JR. Identification of protein-protein interactions with glutathione-S-transferase fusion proteins in Protein-Protein Interaction: A Molecular Cloning Manual. Golemis, Erica, editor. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York: 2002. p. 37-58.2002
- Fröblius AC, Kanost MR, Gotz P, Vilcinskas A. Isolation and characterization of novel inducible serine proteinase inhibitors from larval hemolymph of the greater wax moth *Galleria mellonella*. *Eur J Biochem* 2000;267:2046–2053. [PubMed: 10727944]
- Gorman MJ, Wang Y, Jiang H, Kanost MR. *Manduca sexta* hemolymph proteinase 21 activates prophenoloxidase-activating proteinase 3 in an insect innate immune response proteinase cascade. *J Biol Chem* 2007;282:11742–11749. [PubMed: 17317663]
- Jiang H, Wang Y, Kanost MR. Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. *Insect Mol Biol* 1996;5:31–38. [PubMed: 8630533]
- Jiang H, Wang Y, Kanost MR. Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter. *Proc Natl Acad Sci USA* 1998;95:12220–12225. [PubMed: 9770467]
- Jiang H, Wang Y, Yu XQ, Kanost MR. Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*. A bacteria-inducible serine proteinase containing two clip domains. *J Biol Chem* 2003a;278:3552–3561. [PubMed: 12456683]
- Jiang H, Wang Y, Yu XQ, Zhu Y, Kanost MR. Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem Mol Biol* 2003b;33:1049–1060. [PubMed: 14505699]
- Laemmli MK. Cleavage of structural proteins during the assembly of the head of bacteria-phage T4. *Nature (Lond)* 1970;227:680–685. [PubMed: 5432063]
- Leclerc V, Pelte N, El Chamy L, Martinelli C, Ligoxygakis P, Hoffmann JA, Reichhart JM. Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Rep* 2006;7:231–235. [PubMed: 16322759]
- Lee E, Linder ME, Gilman AG. Expression of G-protein α subunits in *Escherichia coli*. *Meth Enzymol* 1994;237:146–163. [PubMed: 7934993]
- Ling E, Yu XQ. Cellular encapsulation and melanization are enhanced by immulectins, pattern recognition receptors from the tobacco hornworm *Manduca sexta*. *Dev Comp Immunol* 2006;30:289–299. [PubMed: 16023723]
- Liu H, Hiravanichpaisal P, Cerenius L, Lee BL, Soderhall I, Soderhall K. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* Infection in a crustacean, *Pacifastacus leniusculus*. *J Biol Chem* 2007a;282:33593–33598. [PubMed: 17855335]
- Liu L, Mushero N, Hedstrom L, Gershenson A. Short-lived protease serpin complexes: partial disruption of the rat trypsin active site. *Protein Sci* 2007b;16:2403–2411. [PubMed: 17962402]
- Lu Z, Jiang H. Regulation of phenoloxidase activity by high- and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*. *Insect Biochem Mol Biol* 2007;37:478–485. [PubMed: 17456442]
- Matova N, Anderson KV. Rel/NF-kappaB double mutants reveal that cellular immunity is central to *Drosophila* host defense. *Proc Natl Acad Sci USA* 2006;103:16424–16429. [PubMed: 17060622]
- Michel K, Budd A, Pinto S, Gibson TJ, Kafatos FC. *Anopheles gambiae* SRPN2 facilitates midgut invasion by the malaria parasite *Plasmodium berghei*. *EMBO Rep* 2005;6:891–897. [PubMed: 16113656]
- Michel K, Suwanchaichinda C, Morlais I, Lambrechts L, Cohuet A, Awono-Ambene PH, Simard F, Fontenille D, Kanost MR, Kafatos FC. Increased melanizing activity in *Anopheles gambiae* does not affect development of *Plasmodium falciparum*. *Proc Natl Acad Sci USA* 2006;103:16858–16863. [PubMed: 17065316]

- Mullen LM, Goldsworthy GJ. Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. *J Insect Physiol* 2006;52:389–398. [PubMed: 16413931]
- Osta MA, Christophides GK, Kafatos FC. Effects of mosquito genes on *Plasmodium* development. *Science* 2004;303:2030–2032. [PubMed: 15044804]
- Rawlings ND, Tolle DP, Barrett AJ. Evolutionary families of peptidase inhibitors. *Biochem J* 2004;378:705–716. [PubMed: 14705960]
- Schnitger AK, Kafatos FC, Osta MA. The melanization reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. *J Biol Chem* 2007;282:21884–21888. [PubMed: 17537726]
- Schwarzenbach GA, Ward PI. Phenoloxidase activity and pathogen resistance in yellow dung flies *Scathophaga stercoraria*. *J Evol Biol* 2007;20:2192–2199. [PubMed: 17956383]
- Shrivastava B, Ghosh AK. Protein purification, cDNA cloning and characterization of a proteinase inhibitor from the Indian tasar silkworm, *Antheraea mylitta*. *Insect Biochem. Mol Biol* 2003;33:1025–1033.
- Suderman RJ, Andersen SO, Hopkins TL, Kanost MR, Kramer KJ. Characterization and cDNA cloning of three major proteins from pharate pupal cuticle of *Manduca sexta*. *Insect Biochem Mol Biol* 2003;33:331–343. [PubMed: 12609518]
- Swanson R, Raghavendra MP, Zhang W, Froelich C, Gettins PG, Olson ST. Serine and cysteine proteases are translocated to similar extents upon formation of covalent complexes with serpins. Fluorescence perturbation and fluorescence resonance energy transfer mapping of the protease binding site in CrmA complexes with granzyme B and caspase-1. *J Biol Chem* 2007;282:2305–2313. [PubMed: 17142451]
- Tong Y, Jiang H, Kanost MR. Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *J Biol Chem* 2005;280:14932–14942. [PubMed: 15695806]
- Tong Y, Kanost MR. *Manduca sexta* serpin-4 and serpin-5 inhibit the prophenol oxidase activation pathway: cDNA cloning, protein expression, and characterization. *J Biol Chem* 2005;280:14923–14931. [PubMed: 15695807]
- Volz J, Muller HM, Zdanowicz A, Kafatos FC, Osta MA. A genetic module regulates the melanization response of *Anopheles* to *Plasmodium*. *Cell Microbiol* 2006;8:1392–1405. [PubMed: 16922859]
- Wang Y, Jiang H. Reconstitution of a branch of the *Manduca sexta* prophenoloxidase activation cascade *in vitro*: snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor. *Insect Biochem Mol Biol* 2007;37:1015–1025. [PubMed: 17785189]
- Xu, Wen-Yue; Jian, Z.; Tao-Li, Z.; Fu-Sheng, H.; Jian-Hua, D.; Ying, W.; Zhong-Wen, Q.; Li-Sha, X. *Plasmodium yoelii*: contribution of oocysts melanization to natural refractoriness in *Anopheles dirus*. *Exp Parasitol* 2007;116:433–439. [PubMed: 17416360]
- Yu XQ, Gan H, Kanost MR. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma prophenol oxidase. *Insect Biochem Mol Biol* 1999;29:585–597.
- Yu XQ, Jiang H, Wang Y, Kanost MR. Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem Mol Biol* 2003;33:197–208. [PubMed: 12535678]
- Yu XQ, Kanost MR. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. *J Biol Chem* 2000;275:37373–37381. [PubMed: 10954704]
- Yu XQ, Kanost MR. Immulectin-2, a pattern recognition receptor that stimulates hemocyte encapsulation and melanization in the tobacco hornworm, *Manduca sexta*. *Dev Comp Immunol* 2004;28:891–900. [PubMed: 15183030]
- Yu XQ, Ling E, Tracy ME, Zhu Y. Immulectin-4 from the tobacco hornworm *Manduca sexta* binds to lipopolysaccharide and lipoteichoic acid. *Insect Mol Biol* 2006;15:119–128. [PubMed: 16640722]
- Yu XQ, Tracy ME, Ling E, Scholz FR, Trenczek T. A novel C-type immulectin-3 from *Manduca sexta* is translocated from hemolymph into the cytoplasm of hemocytes. *Insect Biochem Mol Biol* 2005;35:285–295. [PubMed: 15763465]

- Zhang G, Lu ZQ, Jiang H, Asgari S. Negative regulation of prophenoloxidase (proPO) activation by a clip-domain serine proteinase homolog (SPH) from endoparasitoid venom. *Insect Biochem Mol Biol* 2004;34:477–483. [PubMed: 15110869]
- Zhang J, Huang FS, Xu WY, Song P, Duan JH, Yang S, Qiu ZW. *Plasmodium yoelii*: correlation of up-regulated prophenoloxidase and phenoloxidases with melanization induced by the antimalarial, nitroquine. *Exp Parasitol* 2008;118:308–314. [PubMed: 17936755]
- Zhao P, Li J, Wang Y, Jiang H. Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *Manduca sexta* phenoloxidase. *Insect Biochem Mol Biol* 2007;37:952–959. [PubMed: 17681234]
- Zhu Y, Wang Y, Gorman MJ, Jiang H, Kanost MR. *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J Biol Chem* 2003;278:46556–46564. [PubMed: 12966082]

Fig. 1. Purification and characterization of *M. sexta* cationic protein CP8

(A) Far western blot analysis of *M. sexta* larval plasma proteins that interact with recombinant IML-3 (rIML-3). Cell-free plasma (2 μ l) from *M. sexta* naïve larvae was analyzed on SDS-PAGE, and plasma proteins were stained with Coomassie Brilliant Blue (lane 1) or transferred to a nitrocellulose membrane. Then the membrane was probed with the His-tagged rIML-3 (lane 2). Binding of rIML-3 to plasma proteins was detected by monoclonal antibody to the His-tag. Lane M is protein ladder. The arrowhead and arrow indicate CP8 and the 14-kDa protein (lysozyme) respectively, which interact with rIML-3. (B) Purification scheme for CP8. 50% AS = 50% ammonium sulfate. Far western blot was used to monitor the desired proteins at each step of purification. (C) Analysis of native and recombinant CP8 by SDS-PAGE.

Recombinant (lane 1) and native CP8 (lane 2) (each at 0.5 μ g) were analyzed on 15% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Lane M is protein ladder, and the arrowhead indicates CP8. (D) MALDI-TOFF analysis of CP8 purified from plasma. The main peak of CP8 has a molecular weight of 9094 ± 2 kDa. Insulin was used as an internal molecular weight standard.

A

1 GAGTGTGCTTTCCACCAGCCATTACATTGGTAGCTGCAGATCAGTTAGTACGAGTAACT
61 CCTCTCCTTATTCAAATGAAAGCTGTCGTAGTACTAGTTGTGCTGGCGTGCGCCATGGC
M K A V V V L V V L A C A M A
121 GGTGGTGTACGGAGACTTGGTGTGCGGCTCAAAC TACTGCAAGCAACACCC TTGCGGGCTC
16 V V Y G D L V (C) G S N Y (C) K Q H P (C) G S
181 GCCCATCGCTCAGTCGAGCTGTCGCTCCCTTCCGCTCTACCGCGCCAAACACGCCGGCAA
36 P I A Q S S (C) R S P S V Y R A K H A G K
241 ATGCGCCTGCTGCCCTGCCTGCGTTACTATGCTTGGTGAGAACGCCGCTTGTAAGACCTA
56 (C) A (C) (C) P A (C) V T M L G E N A A (C) K T Y
301 CAGCAAAGAGCTTGGAGAGACCCCATCCGCCATCTGTCGTGACCTCTTTAAGTGCCGTGAA
76 S K E L G E T P S A I (C) R D P L K (C) L N
361 CGGTGTCTGTACCAGGGTCGCTCCTAGACACTAAGTACTTACAA TTAATAA TATAAAATG
96 G V (C) T R V A P R H *
421 TTAATGAAAAATAAAAAGCATCAAAGTCTTAAATAAACTAATACATAACATCCGCCGTA
481 ATTAAAAAAAAAAAAAAAAAAAAA

B

M. sexta MKAVVVLVVLACAMAVVYGDLVCGSNYCKQHPCGSPITAQ--SSCRSPSVYRAK---HAGKC
A. mylitta MKAVITLLFLACILVVITYGDLICGTNYCKDHPCISPIAR--ASCRSPATYRAN---HSGKC
P. sitchensis MKAALVLFVLACALALASSDLVCGSKYCKQNPICSAPTITSNKCRSPA VYRPN---HAGKC
B. mori -----MRCVTRYGALVCGTDYCEKNPICIQPF--LVCPKNTTEHRAR---HAGKC
B. mandarina -----MRCAAAYGALVCGTDYCEKNPICIQPF--LVCPKNTTEHRAR---HAGKC
G. mellonella -----DLVXGTNFXKNNPX--STRVAANSXRSPSVYRQXXXXHAKKA

***** * * * ** ***** ***** * ***** ***

M. sexta ACCPACVTMLGENAACKTYSKELGETPSAICRDPLKCLNGVCTRVAPRH (100%)
A. mylitta ACCPACVTLLRERAAACKTYSKEIGETPSAVCQEPLKCLNGVCTKVTPRR (73%)
P. sitchensis ACCPACVTLLSEGASCKHYSKEMGETPSAVCREPLRCLKGVCINAPLRN (67%)
B. mori ACCPACVTLLGEGATCKIYSKELGETPSAVCKEPLCKIKRVCTKLKLV (61%)
B. mandarina ACCPACVTLLDEGATCKIYSKELGETPSAVCKEPLCKIKRVCTKLKLV (59%)
G. mellonella AXPPAVVTLL (50%)

Fig. 2. The nucleotide and deduced amino acid sequences of CP8 and alignment of CP8 with homologous proteins

(A) The nucleotide and deduced amino acid sequences of CP8. The deduced amino acid sequence of CP8 is shown below the cDNA sequence. The amino-terminal sequence of the mature CP8 was determined by Edman degradation and is underlined. Cysteine residues are circled. The polyadenylation sequence AATAAA is double-underlined. (B) Alignment of CP8 with homologous proteins. The deduced amino acid sequence of CP8 was aligned with *A. mylitta* AmFPI-1 (accession number: **B0JFB8**), *G. mellonella* ISPI-1 (accession number: **P81905**), and homologous proteins from *P. sitchensis* (accession number: **ABK25652**), *B. mori* (accession number: **AY655143**) and *B. mandarina* (accession number: **EF126182**). Residues conserved in all five proteins but *G. mellonella* ISPI-1 are marked with '*' above the alignment. Conserved cysteine residues are indicated by boxes. Identities between CP8 and other proteins are included in the parentheses.

Fig. 3. Expression of CP8 in *M. sexta* larvae

(A) Real-time PCR analysis of CP8 mRNA in tissues of naïve larvae. Real-time PCR was performed as described in the Materials and Methods. Expression level in the naïve hemocytes was used as the calibrator. (B) Real-time PCR analysis of CP8 mRNA in fat body of immune-challenged larvae. Day 2 fifth instar *M. sexta* naïve larvae were injected with saline, formaldehyde-killed *E. coli* (10^8 cells per larva), *M. luteus* (100 μ g per larva), or yeast (*S. cerevisiae*, 10^7 cells per larva), and fat body was collected at 24 h post-injection for preparation of total RNAs. Real-time PCR was performed the same as in (A). Expression level in fat body of *M. luteus*-injected larvae was used as the calibrator. The significance of difference was calculated using an unpaired t-test program (GraphPad, San Diego, CA). (C) Expression of CP8 protein in hemolymph of immune-challenged larvae. Day 2 fifth instar *M. sexta* naïve larvae were injected with saline, *E. coli*, *M. luteus*, or yeast as described in (B), and hemolymph was collected at 0 h (naïve hemolymph) and 24 h post-injection. Aliquots of plasma (1 μ l, a mixture from at least 4 larvae) were analyzed on 15% SDS-PAGE, and CP8 was identified by immunoblotting using rabbit polyclonal antibody to recombinant CP8. The arrow indicates CP8 in hemolymph.

Fig. 4. CP8 does not have an effect on serine proteinase activity

Native CP8 purified from plasma, renatured recombinant CP8, or BSA (each at 0.5 μ g) was mixed with a serine proteinase (50ng trypsin, 100ng chymotrypsin, 200ng elastase, 200ng subtilisin A, 200ng proteinase K, 200ng *S. griseus* proteinase, or 10ng PAP-3) in a total of 20 μ l assay buffer (100mM Tris-HCl, 100mM NaCl, 1mM CaCl₂, pH7.5) and incubated at room temperature for 30 min. Then a specific peptide substrate (200 μ l, 50 μ M) was added and proteinase activity was monitored every min for 30 min. The bars represent the means of three individual measurements $\pm$ SEM.

Fig. 5. CP8 stimulates proPO activation in plasma

Aliquots of naïve plasma (#31) (2 µl each) were incubated with heat-treated (65°C, 30 min) native CP8 purified from plasma or BSA (0.1 or 0.5 µg) in a total of 10 µl TBS for 20 min (A) or 30 min (B) at room temperature, or with TBS alone (Control), CP8/*M. luteus* mixture (0.5 µg each) or BSA/*M. luteus* mixture (0.5 µg each) for 10 min at room temperature (C), or pre-activated with *M. luteus* (0.5 µg) for 5 min at room temperature and then incubated with CP8 or BSA (0.5 µg each) for another 5 min (totally 10 min) at room temperature (C). Finally, L-dopamine substrate (200 µl, 2mM) was added to each sample and PO activity was monitored with time at 470nm for 30 min. The bars represent the means of four individual measurements $\pm$ SEM. The significance of difference was calculated using an unpaired t-test program (GraphPad, San Diego, CA). Assays in the same panel (A, B or C) were performed at the same time, while assays in different panels were performed at different times. Purified CP8 (lanes 1–2, 0.1 and 0.2 µg, respectively), aliquots of naïve plasma #31 (lanes 3–4, 0.5 and 1 µl, respectively), naïve plasma #2 (lane 5, 1 µl) and naïve plasma #38 (lane 6, 1 µl) were also analyzed by SDS-PAGE, and CP8 was identified by immunoblotting using rabbit polyclonal antibody to recombinant CP8 (D). The band intensities of the Western blot in (D) were measured using Kodak Digital Science 1D gel analysis software and normalized to the amount of CP8 (0.2 µg) in lane 2 (E).