Insect Molecular Biology

Insect Molecular Biology (2017) **26**(5), 552–563 doi: 10.1111/imb.12318

Activation of *Aedes aegypti* prophenoloxidase-3 and its role in the immune response against entomopathogenic fungi

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Abstract

Serine protease cascade-mediated melanization is an important innate immune response in insects and crustaceans, which involves the proteolytic activation of prophenoloxidase (PPO). In this study, we investigated the role of *Aedes aegypti* PPO3 in antifungal immune defence. We expressed and purified recombinant PPO3 (rPPO3) in *Escherichia coli* and demonstrated that rPPO3 was activated by ethanol and, to a lesser extent, by cetylpyridinium chloride. In the presence of Cu^{2+}, rPPO3 exhibited enzyme activity. Immunoblot results revealed that the rPPO3 was cleaved by the haemolymph from immune-challenged mosquitoes or purified *Ostrinia furnacalis* serine protease 105 *in vitro*. The cleaved rPPO3 converted dopamine to toxic intermediates that killed fungal conidia of *Beauveria bassiana in vitro*. In mosquitoes challenged with *Be. bassiana*, cleavage of rPPO3 produced a 50 kDa phenoloxidase (PO) fragment. Further analysis revealed that the survival rate of mosquitoes with fungal infection increased significantly following injection of rPPO3 into the haemocoel. Taken together, our results suggest that proteolytic cleavage of the mosquito PPO3 plays an important role in the antifungal immune response. This has led to a better understanding of the mechanism of PPO activation in the mosquito and the role of melanization in the antifungal immune response.

Keywords: prophenoloxidase, melanization, antifungal immunity, mosquito, insect immunity.

Introduction

Haematophagous insects act as vectors of human diseases. The mosquito *Anopheles gambiae* transmits malaria, a devastating disease that causes nearly half a million deaths every year. The mosquito *Aedes aegypti* is a major vector of flaviviruses, including dengue, yellow fever, chikungunya and Zika viruses. In 2015, Zika virus emerged for the first time in Brazil and quickly spread to more than 20 countries and regions (Musso & Gubler, 2016). These recent Zika virus outbreaks indicate that vector control is urgently needed.

In past decades, chemical pesticides were widely used to control insect pests, but their use caused serious problems with insecticide resistance and environmental pollution (Wang *et al.*, 2013). A variety of entomopathogenic fungi are currently used as biological pesticides for mosquito control (Read *et al.*, 2009). The infection of insects by entomopathogens results in complex reactions and, thus, deciphering the molecular interactions between microbes and their insect hosts would help the efficacy of biocontrol products.

Insects lack an adaptive immune system and rely on their innate immune responses to combat microbial infections (Jiang *et al.*, 2010). Melanization, a prominent immune response in arthropods including insects (Cerenius *et al.*, 2008; Scherfer *et al.*, 2008), plays an important role in wound healing and pathogen sequestration in diverse species, including *Penaeus monodon*,

First published online 27 May 2017.

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Manduca sexta and *Drosophila melanogaster* (Tang *et al*., 2006; Zhao *et al*., 2007; Sutthangkul *et al*., 2015). The antipathogen melanization mechanism is also present in mosquitoes. *Armigeres subalbatus* and *An. gambiae* uses melanization in antibacterial (Hillyer *et al*., 2003) and antifungal (Yassine *et al*., 2012) immune responses respectively. Similarly, melanization controls these events in *Ae. aegypti* (Zou *et al*., 2008).

The melanization reaction is initiated by recognition of molecular patterns on the microbe surface by the pathogen pattern-recognition proteins, such as β-1,3-glucan recognition protein and peptidoglycan recognition protein. It has been demonstrated that in *M. sexta*, an enzyme cascade composed of serine proteases (SPs) containing regulatory clip domains (CLIPs) is activated to convert prophenoloxidase (PPO) to phenoloxidase (PO) (Takahashi *et al*., 2014). PPO can also be activated *in vitro* by chemicals such as ethanol or detergents (Asada & Sezaki, 1999; Li *et al*., 2012). The central event of melanization is proteolytic activation of PPO. PPO cleavage generates the mature enzyme PO. To prevent spontaneous melanization, the PPO activation cascade is tightly regulated by serine protease inhibitors (serpins), such as *M. sexta* serpin-3 and serpin-6 (Zhu *et al*., 2003).

Genome analysis has revealed that the number of melanization-related genes has undergone a large expansion in mosquitoes. For example, there are nine and 10 PPO genes in *An. gambiae* and *Ae. aegypti*, respectively. There has also been an expansion of CLIPs and serpins (Zou *et al*., 2010). This suggests an increased complexity of melanization reactions during antipathogen defences in these insects. It has been found that in *Ae. aegypti*, there are at least two different pathways, immune and tissue melanization, that are controlled by distinct modules of CLIPs and serpins. The immune melanization proteases (IMP-1 and IMP-2) and serpin-1 mediate immune melanization. Tissue melanization is controlled by CLIPB8, IMP-1 and serpin-2 (Zou *et al*., 2010).

Previously, we found that the mRNA abundance of *PPO3* specifically increased after fungus *Beauveria bassiana* infection. PPO3 was cleaved in the haemolymph, and this reaction was regulated by an enzyme CLSP2, which consists of C-type lectin and SP domains from *Ae. aegypti* (Wang *et al*., 2015). However, the mechanism of PPO3 activation and its function in response to pathogens have remained unknown. In this study, we expressed *Ae. aegypti* PPO3 in *Escherichia coli* and used the recombinant protein (rPPO3) to study PPO3 activation mechanism. The function of PPO3 in the antifungal response was also investigated. Our results provide a new insight into the relationship between PPO cleavage and function.

Results

Purification of rPPO3 protein from Es. coli

To understand the biochemical properties and functions of *Ae. aegypti* PPO3, we expressed rPPO3 with N- and C-terminal 6 × His tags in *Es. coli*. The soluble rPPO3 was purified using nickel-changed affinety resin (Ni-NTA). The purified protein migrated as a single band with the expected molecular mass of approximately 79 kDa (Fig. 1A). Trypsinolytic peptide mass fingerprint analysis of the band revealed 22 matching peptides, representing 40.2% of the overall sequence of PPO3. Immunoblot analysis demonstrated that rPPO3 can be recognized by both the anti-His and the anti-PPO3 antibodies (Fig. 1B).

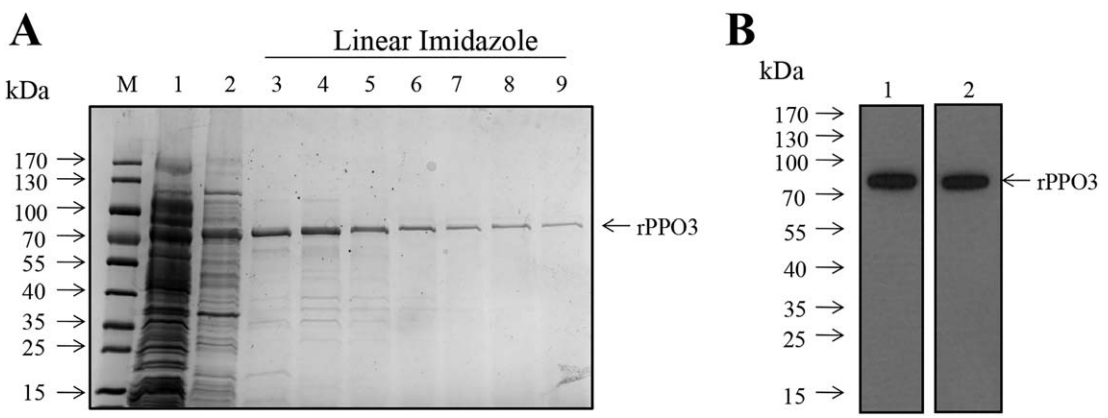

Figure 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of recombinant prophenoloxidase-3 (rPPO3). (A) Purified protein samples were analysed by SDS-PAGE followed by Coomassie Blue staining. rPPO3 bands are indicated by arrows. M, prestained protein ladder; 1, the supernatant following lysis of *Escherichia coli*; 2, supernatant fraction precipitated with ammonium sulphate and re-suspended; 3–6, fractions eluted with 100 mM imidazole; 7–9, fractions eluted with 200 mM imidazole. (B) Purified product was detected by immunoblotting with anti-His antibodies and anti-PPO3 antibodies. 1, detection with anti-His antibodies; 2, detection with anti-PPO3 antibodies.

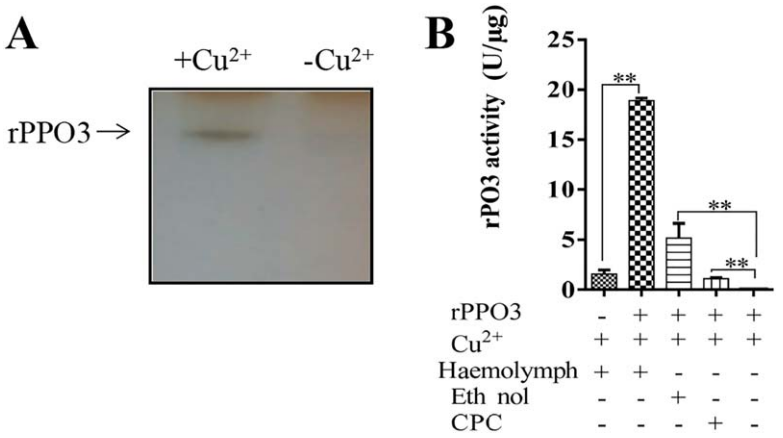

Figure 2. Mosquito recombinant prophenoloxidase-3 (rPPO3) exhibits enzyme activity. (A) Detection of rPPO3 by native gel electrophoresis. The arrow indicates the band representing rPPO3 in the presence of Cu^{2+}. (B) Purified rPPO3 can be activated by many factors. Haemolymph, ethanol and cetylpyridinium chloride (CPC) were used separately to activate rPPO3. Error bars represent means ± SD from three replicates. ** $P < 0.01$. [Colour figure can be viewed at wileyonlinelibrary.com]

Assay of rPPO3 enzyme activity

A native gel assay was used to examine rPPO3 in the presence (+) or absence (–) of Cu^{2+}. Dopamine was used as a substrate in the evaluation of rPPO3 activation (Asada & Sezaki, 1999). Before loading rPPO3 onto the gel, Cu^{2+} was added to the protein to a final concentration of 0.5 mM, whereas the control group did not contain Cu^{2+}. The gel was incubated in 10 mM dopamine for 10 min and developed in 30% ethanol until protein bands appeared. In the presence of Cu^{2+}, there was a clear band representing rPPO3; however, this band was not detected in the absence of Cu^{2+} (Fig. 2A). This result indicated that the rPPO3 had enzyme activity.

In order to assay the effect of Cu^{2+} on the enzymatic activity, rPPO3 was treated with different concentrations of Cu^{2+} (0–4 mM) for 5 min. One group was treated with 30% ethanol for 5 min to activate rPPO3, and a second group was treated with 1 μl fungal-challenged mosquito haemolymph. The results showed that following ethanol activation, rPPO3 did not exhibit any enzyme activity until Cu^{2+} was added. PO activity gradually increased, reaching 6 U/μg when the concentration of Cu^{2+} was 0.5 mM (Fig. S1A). After incubation with haemolymph, rPPO3 exhibited high enzyme activity even in the absence of Cu^{2+}. However, PO activity increased up to 25 U/μg when the Cu^{2+} was added at a concentration of 0.1 or 0.2 mM (Fig. S1B).

For further comparison of the different factors required to activate rPPO3, rPPO3 was incubated with 0.5 mM Cu^{2+} for 5 min at room temperature and treated with 1 μl fungal-challenged mosquito haemolymph, 30% final concentration of ethanol, or 10 μl cetylpyridinium chloride (CPC; 1 ng/μl) for 10 min. The results indicated that after activation by 30% ethanol or CPC, enzyme activity of rPPO3 was not very high, whereas haemolymph activated rPPO3 more efficiently, reaching 20 U/μg (Fig. 2B). These results showed that rPPO3 expressed in *Es. coli* can be activated by different factors. Cu^{2+} is required for activity of rPPO3, but high concentrations of Cu^{2+} appears to inhibit activity.

Cleavage of rPPO3 by haemolymph and SP in vitro

PO is a key enzyme involved in melanization. To initiate melanization, PPO needs to be cleaved at a specific site by a CLIP to form PO. To assess whether rPPO3 can be cleaved *in vitro* by an active SP from mosquito haemolymph, approximately 1 μg rPPO3 and 1 μl fungal-challenged mosquito haemolymph were combined, incubated at room temperature for 60 min, and analysed by immunoblot using anti-His antibodies. The results showed that the band representing rPPO3 disappeared after incubation with mosquito haemolymph, and a weak band around 60 kDa appeared. The band around 50 kDa representing rPO3 was apparent, in addition to a band around 20 kDa (Fig. 3A). Our results suggested that the 60 kDa band may be an intermediate product, which is further cleaved into a band of 50 kDa. In the control group, we first added 1 μl protease inhibitor cocktail to the haemolymph, incubated for 15 min on ice, and then added rPPO3. This resulted in a clear rPPO3 band and only a weak rPO3 band as well as a 60 kDa band (Fig. 3A). These results indicate that rPPO3 can be cleaved by fungal-challenged mosquito haemolymph.

Ostrinia furnacalis SP105 (*Of*SP105) can reportedly cleave and activate *D. melanogaster* PPO1 (Chu *et al*., 2017). Here we examined whether *Ae. aegypti* rPPO3 (*Aa*rPPO3) can also be cleaved by *Of*SP105. Firstly, recombinant proSP105 was activated by Factor Xa as described previously (Chu *et al*., 2015), and approximately 1.0 μg rPPO3 was added and incubated at room temperature for 1 h. In the control samples, *Dm*PPO1 was added as a substrate for SP105 (Chu *et al*., 2017), and fungal-challenged mosquito haemolymph was used to cleave *Aa*rPPO3. The immunoblot results showed that *Of*SP105 cleaves rPPO3 into a PO band and an N-terminal band (Fig. 3B). In the control group, without

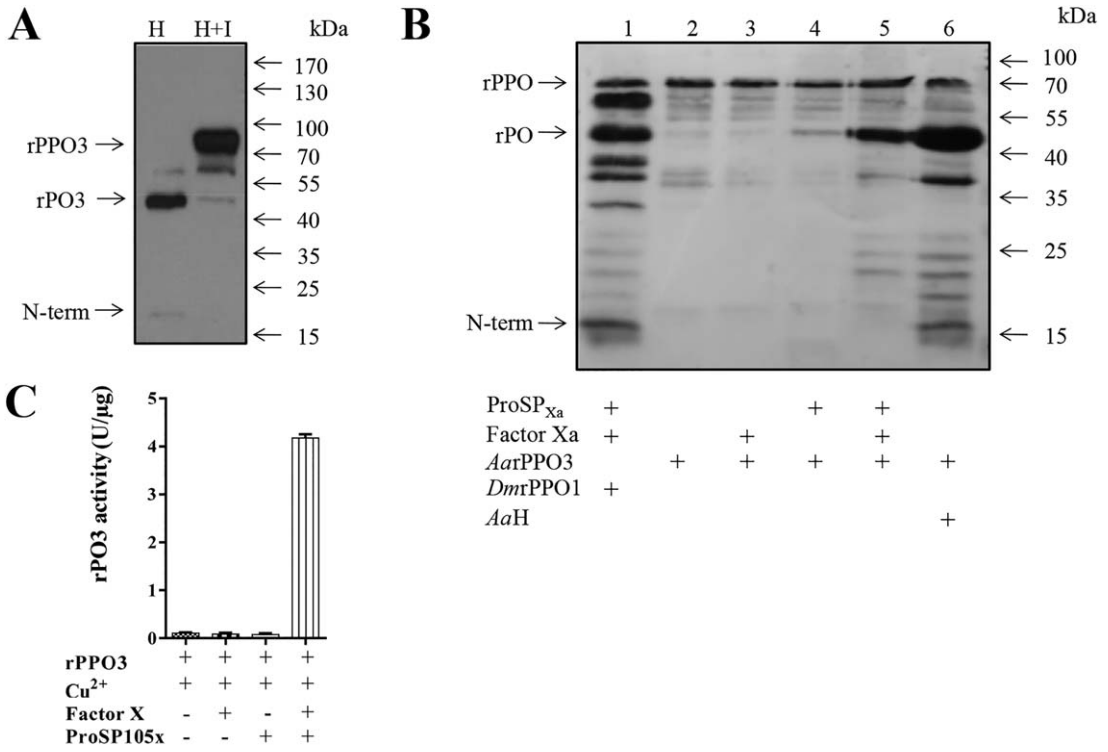

Figure 3. Cleavage of recombinant prophenoloxidase-3 (rPPO3) by haemolymph and a serine protease *in vitro*. (A) Cleavage of rPPO3 by fungal-challenged mosquito haemolymph was analysed by immunoblot using anti-PPO3 antibodies. The rPPO3, 50 kDa recombinant phenoloxidase-3 (rPO3) and N-terminal (N-term) bands are indicated by arrows. (B) Activation of purified *Aedes aegypti* rPPO3 (*Aa*rPPO3) by *Ostrinia furnacalis* serine protease 105 (*Of*SP105). The samples were analysed by immunoblot using anti-His antibody. (C) The activity of rPPO3 cleaved by activated *Of*SP105. Purified *Aa*rPPO3 was mixed with Factor Xa, proSP105$_{Xa}$ or Factor Xa activated proSP105$_{Xa}$. Error bars represent means ± SD from three replicates. H, fungal-challenged mosquito haemolymph; H+I, fungal-challenged mosquito haemolymph with protease inhibitor. proSP105$_{Xa}$, serine protease 105 from *O. furnacalis* without activity; Factor Xa, an enzyme of the coagulation cascade from New England BioLabs; *Dm*rPPO1, purified recombinant protein from *Drosophila melanogaster* PPO1; *Aa*H, haemolymph from fungal-challenged *Ae. aegypti* mosquitoes; M, molecular mass standards.

activation by Factor Xa, proSP105 could not cleave rPPO3 (Fig. 3B). Meanwhile, we also investigated whether the activated *Of*SP105 can activate *Aa*rPPO3. The results showed that, in the presence of Factor Xa and proSP105$_{Xa}$, rPPO3 can be activated, yielding enzymatically active PO3 (Fig. 3C). It appears that activated SP can cleave and activate rPPO3, although several regulatory units have been implicated in *Ae. aegypti* and *An. gambiae* (Zou *et al.*, 2010; An *et al.*, 2011; Zhang *et al.*, 2016), and their role in the direct cleavage of PPO3 will be worth exploring.

Identification of the PPO3 cleavage site

To determine the PPO3 cleavage site, we first analysed the sequence of the protein fragment generated upon PPO3 activation. Recombinant PPO3 was incubated with fungal-challenged mosquito haemolymph for 1 h, and Ni-NTA agarose was then used to purify the protein fractions using the His tag. The protein fractions were separated on a 4–15% Mini-PROTEAN TGX (San Diego, UAS) gel. In addition to the 50 kDa band, another high molecular mass complex was also detected in the eluted fraction (Fig. 4A). These two fragments were excised for identification by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF-MS). The names and GenBank accession numbers for the proteins identified in the high molecular mass complex are listed in Table S1. Our results indicate that the high molecular mass complex contains many proteins in addition to PPO3. We detected peptides identified as SPs, which is notable because many studies have reported that SPs play important roles in the cascade of PPO activation (Kanost & Jiang, 2015). In the silkworm *Bombyx mori*, haemolymph melanization involves the formation of a high molecular mass complex (Clark & Strand, 2013). We speculate that this protein complex in *Ae. aegypti* may also be involved in melanization. The exact function of this high molecular mass complex in melanization in *Ae. aegypti* will be assessed in future studies.

The peptide sequence of the 50 kDa band was highly similar to that of the C-terminus of PPO3 according to LC/Q-TOF-MS (Fig. 4B). We also determined the N-terminal sequence of the 50 kDa band (Fig. 4A). Both results demonstrate that the 79 kDa PPO3 was cleaved into a 50

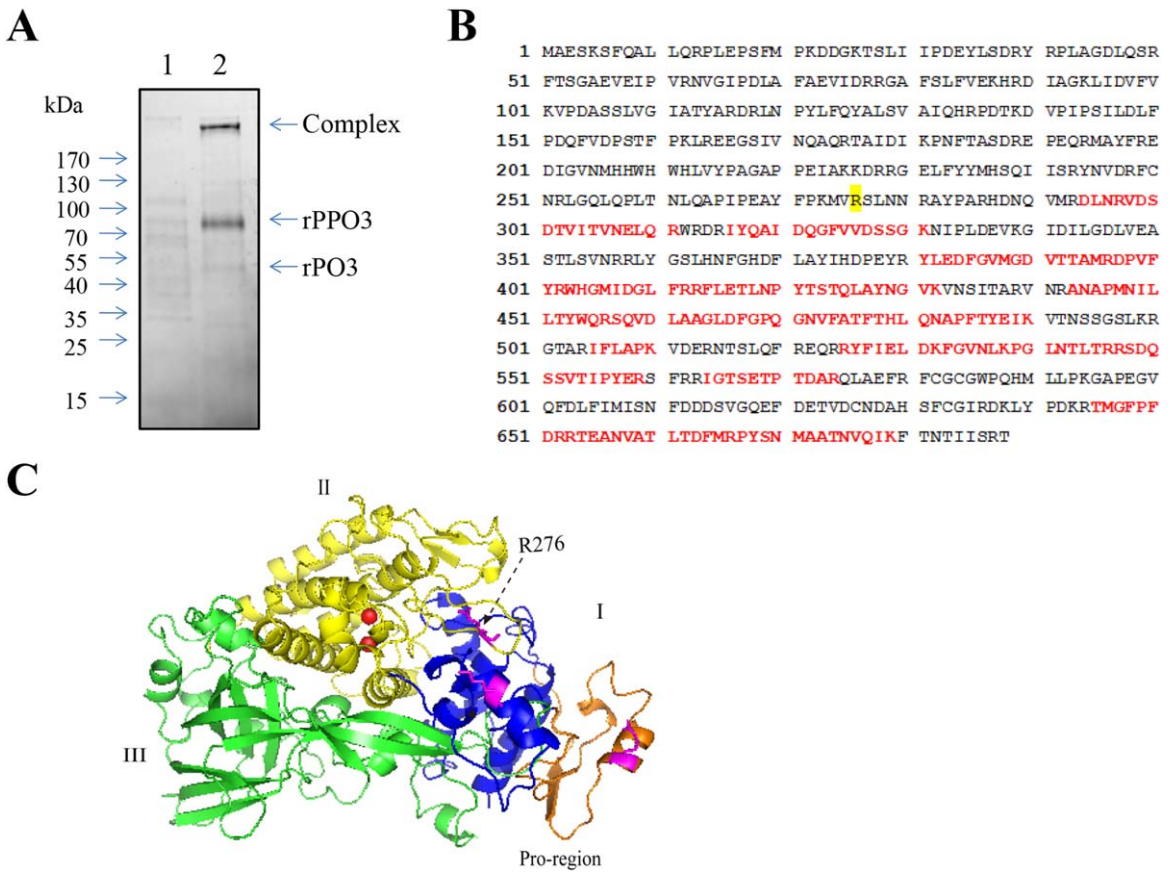

Figure 4. Identification of the prophenoloxidase-3 (PPO3) cleavage site. (A) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (4–15%) of the flow-through (1) and eluted (2) fractions from Nickel-charged affinity resin (Ni-NTA) following incubation of recombinant PPO3 (rPPO3) with haemolymph. The high molecular mass complex, rPPO3 and recombinant phenoloxidase-3 (rPO3) bands are indicated by arrows. (B) Mass spectrometry (MS) results for the rPO3 band. The full-length PPO3 amino acid sequence is shown, and the red letters represent the matched peptides detected by MS. Cleavage site is highlighted with yellow shading. (C) Predicted tertiary structure of *Aedes aegypti* PPO3. The red sphere indicates the Cu^{2+}; domains I–III are represented by blue, yellow and green, respectively; and the arrows indicate the presumed cleavage sites. [Colour figure can be viewed at wileyonlinelibrary.com]

kDa PO at Arg^{276}*Ser^{277} by proteolytic activation. The crystal structures of *M. sexta* PPOs and *An. gambiae* PPO8 were solved previously (Li *et al.*, 2009; Hu *et al.*, 2016). We used *Ag*PPO8 as a template; predicted tertiary structures of *Aa*PPO3 were established using the SWISS-MODEL workspace prediction algorithm. *Aa*PPO3 is 50.8% identical in amino acid sequence to *Ag*PPO8. Quality of the protein models was assessed using the QMEAN Z-score (–1.83; Benkert *et al.*, 2009). The overall structure of *Aa*PPO3 contains two identical subunits, each comprising 688 residues, which form a homodimer in the asymmetrical unit (Fig. S3). The overall structure of *Aa*PPO3, presented in Fig. 4C, includes three domains and a pro-region. Our speculated cleavage site (Arg^{276}*Ser^{277}) is located in domain II (182–420).

Effect of rPPO3 on the growth of Be. bassiana

To examine whether *Aa*rPPO3 has antifungal activity, rPPO3 was cleaved by the activated *Of*SP105 and then incubated with dopamine and *Be. bassiana* conidia expressing green fluorescent protein (GFP). We observed that, in the control groups, without dopamine, activated rPPO3 did not kill fungi conidia (Fig. 5A), and in the absence of activated rPPO3, dopamine also had no antifungal activity (Fig. 5B, C). On the contrary, only a few of the conidia expressing GFP were detected after treatment with activated rPPO3 and dopamine, whereas most of the conidia were dead and did not show fluorescence (Fig. 5D). Thus, our results indicate that the activated rPPO3 can kill fungal conidia in the presence of dopamine.

rPPO3 can be cleaved in mosquitoes during fungal infection

Our analysis suggests that activated rPPO3 can catalyse dopamine to kill fungal conidia *in vitro*, and we wanted to know whether or not rPPO3 performs a similar function in mosquitoes during *Be. bassiana* infection. Our previous results showed that, following fungal

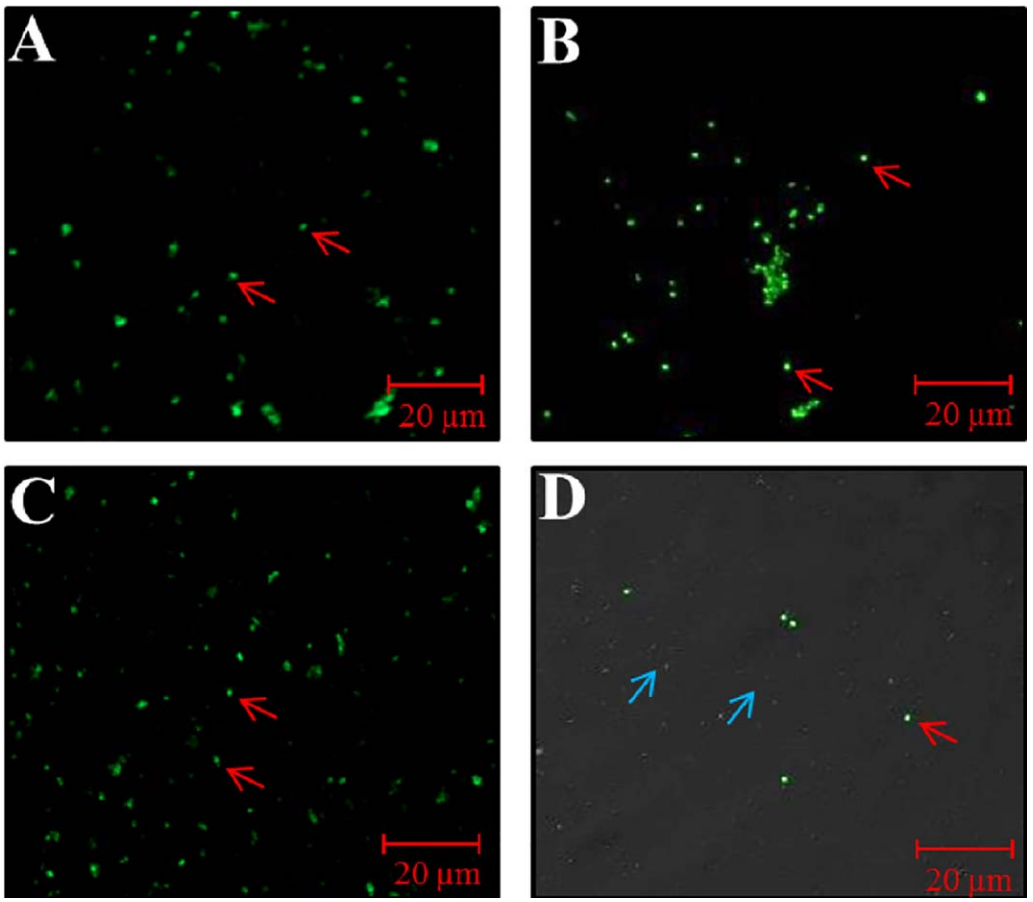

Figure 5. Effect of activated recombinant prophenoloxidase-3 (rPPO3) on the growth of *Beauveria bassiana*. Numbers of conidia expressing green fluorescent protein (GFP) (red arrow) were detected under a fluorescence microscope (A–D). Without dopamine (A), activated rPPO3 cannot kill fungi; dopamine itself (B) does not kill conidia; activated *Ostrinia furnacalis* serine protease 105 also cannot catalyse dopamine to kill fungi (C). After incubating with activated rPPO3 and dopamine, only a few of the conidia expressing GFP (red arrow) and dead fungal conidia without fluorescence (blue arrow) were detected (D). [Colour figure can be viewed at wileyonlinelibrary.com]

infection, PPO3 was cleaved in mosquito haemolymph and a band of approximately 50 kDa was observed in the haemolymph. However, in the samples treated with phosphate-buffered saline (PBS), only the band representing PPO3 was apparent (Wang *et al.*, 2015).

To determine whether rPPO3 can be cleaved *in vivo*, rPPO3 was injected into mosquitoes (approximately 0.1 μg/mosquito) infected with *Be. bassiana* or PBS for 24 h. After 15 min, the mosquito haemolymph was collected and 1× protease inhibitor cocktail was added immediately. The haemolymph was incubated with Ni-NTA agarose for 30 min to purify the His-tagged proteins, and the samples were analysed by immunoblot using anti-His antibodies (Fig. 6A). The results showed that in the sample infected with *Be. bassiana* for 24 h, there was a band of approximately 50 kDa, similar to the rPO3 cleaved by SP or haemolymph *in vitro*. (Fig. 6B). Conversely, in the control group treated with PBS, the rPPO3 band was not cleaved. Therefore, rPPO3 can be cleaved in mosquitoes infected with fungus, suggesting that PPO3 may be involved in the mosquito antifungal immune response.

The effect of PPO3 on fungal infection

To address the role of rPPO3 in the immune response, we investigated the contribution of rPPO3 to the survival of mosquitoes infected with fungi. The mosquitoes were infected with the fungus *Be. bassiana* or PBS and then injected with rPPO3 (approximately 0.1 μg/mosquito). Following treatment, the survival rate of mosquitoes challenged with *Be. bassiana* decreased significantly compared with the control group challenged with PBS (Fig. 7A). However, following injection of rPPO3, the mosquitoes were more resistant to *Be. bassiana* infection, as evidenced by the significant increase in survival rate (Fig. 7A). These results suggest that rPPO3 is involved in the antifungal immune response in mosquitoes.

We further evaluated the effect of PPO3 on fungal infection *in vivo*. The survival rate of PPO3 double-

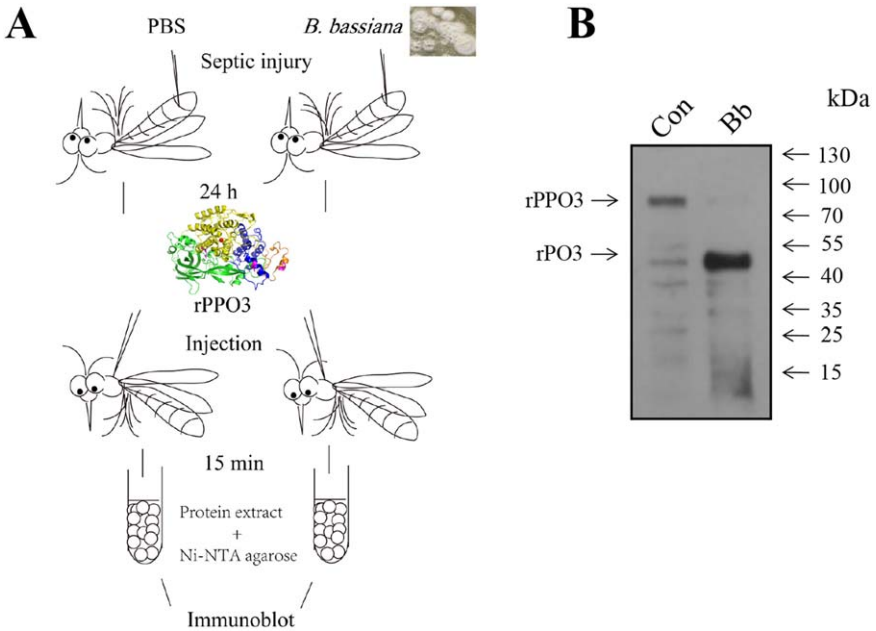

Figure 6. Recombinant prophenoloxidase-3 (rPPO3) can be cleaved in fungal-changed mosquitoes. (A) Experimental procedure. Purified protein was injected into mosquitoes infected with *Beauveria bassiana* or phosphate-buffered saline (PBS). Nickel-charged affinity resin (Ni-NTA) agarose was used to concentrate the His-tagged proteins. (B) Immunoblot was used to test the cleavage of rPPO3 in fungal-challenged mosquitoes by anti-His antibodies. In the mosquitoes infected with *Be. bassiana* (Bb), there is an obvious band representing recombinant phenoloxidase-3 (rPO3), and the band representing rPPO3 disappeared almost completely. Con, mosquitoes challenged with PBS. [Colour figure can be viewed at wileyonlinelibrary.com]

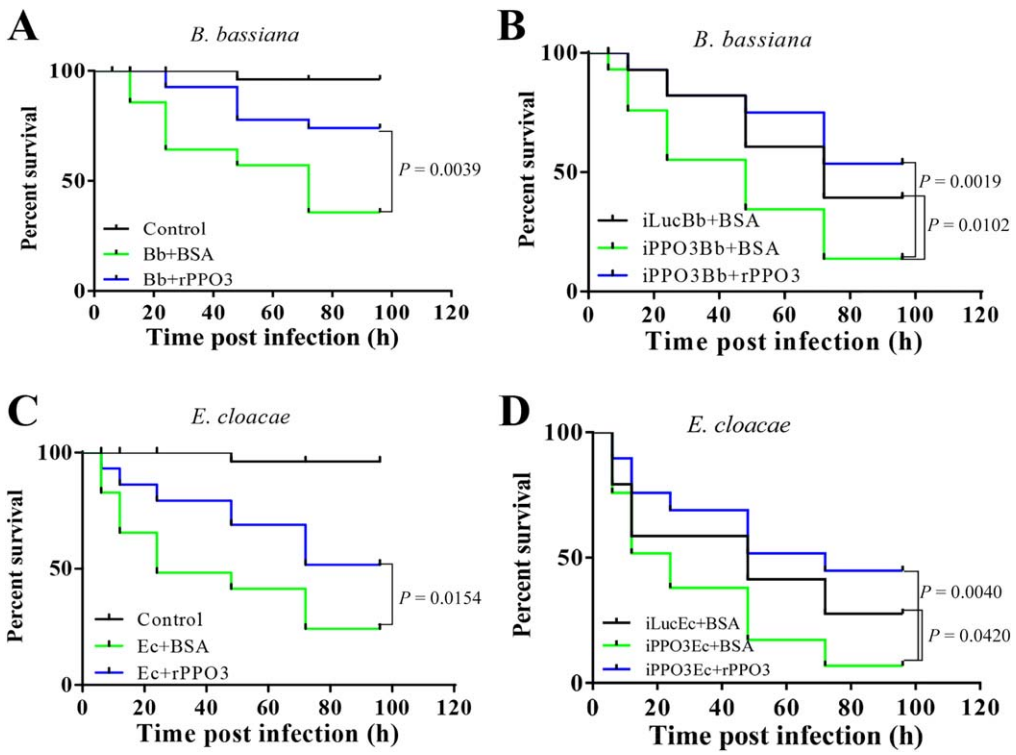

Figure 7. The effect of prophenoloxidase-3 (PPO3) on mosquito survival rate. (A) The survival rate of mosquitoes infected with *Beauveria bassiana* was significantly lower than that of the control ($P < 0.05$). Injection of recombinant PPO3 (rPPO3) increased the survival rate of mosquitoes ($P < 0.05$). (B) PPO3-depleted mosquitoes (iPPO3) were more sensitive to *Be. bassiana* infection ($P < 0.05$). Following rPPO3 injection, the survival rate was partially rescued ($P < 0.05$). (C) The survival rate of mosquitoes decreased after injection with *Enterobacter cloacae*, whereas injection with rPPO3 increased the survival rate ($P < 0.05$). (D) PPO3-depleted mosquitoes were more sensitive to *En. cloacae* infection. After injection of rPPO3, the ability of mosquitoes to respond to *En. cloacae* infection increased ($P < 0.05$). Bb+BSA, mosquitoes infected with *Be. bassiana* (5×10^7) then injected with bovine serum albumin (BSA); Bb+rPPO3, mosquitoes infected with *Be. bassiana* and then injected with rPPO3; iLucBb+BSA, luciferase double-stranded RNA (dsRNA)-treated mosquitoes infected with *Be. bassiana* then injected with BSA; iPPO3Bb+BSA, PPO3 dsRNA-treated mosquitoes infected with *Be. bassiana* and then injected with BSA; iPPO3Bb+rPPO3, PPO3 dsRNA treated mosquitoes infected with *Be. bassiana* and then injected with rPPO3. Identical experiments were performed using *En. cloacae* (Ec). Each experiment was performed in triplicate. [Colour figure can be viewed at wileyonlinelibrary.com]

stranded RNA (dsRNA)-treated mosquitoes was much lower than that of the control groups after infection with *Be. bassiana* (Fig. 7B). However, after the mosquitoes were injected with rPPO3, the survival rate increased significantly (Fig. 7B). An immunoblotting test confirmed the efficiency of PPO3 RNA interference (RNAi; Fig. S2). Meanwhile, we further investigated the effect of PPO3 on the survival rate of mosquitoes during infection by bacterium *Enterobacter cloacae*. The results showed that, after treatment with PPO3 dsRNA, mosquitoes became sensitive to *En. cloacae*. Injection of rPPO3 significantly increased the survival rate (Fig. 7C, D). These results indicate that PPO3 is involved in the antifungal immune response. Moreover, PPO3 may also be involved in the antibacterial immune response.

Discussion

Melanization is a universal defence mechanism in insects and crustaceans (Cerenius & Soderhall, 2004; Liu *et al*., 2007; Cerenius *et al*., 2008). Many studies have reported that melanization is triggered in response to diverse microbes, including parasites (Shin *et al*., 2011), bacteria (Ayres & Schneider, 2008) and fungi (Yassine *et al*., 2012). PPO is a key enzyme involved in the melanization of malaria, and a RUNT-related transcription factor 4 (RUNX4) directly regulated the transcription activation of *PPO* genes (Zou *et al*., 2008). We have also reported that the expression of several *Ae. aegypti PPOs* (PPO1, 3, 5, 8) was up-regulated during microbial challenge and modulated by CLSP2, an upstream factor activated by fungal infection. We found that the cleavage of PPO3 was inhibited by CLSP2 (Wang *et al*., 2015). We speculated that the cleavage of PPO3 may play some roles during fungal infection. However, the detailed mechanism of the mosquito PPO3 activation is still largely unknown. In this study, we expressed and obtained large quantities of rPPO3 for analysis of its activation during *Be. bassiana* infection. Understanding the biochemical properties of PPO3 may provide an explanation as to how melanization participates in the antifungal immune response in mosquitoes.

Type III copper protein families are widely distributed from metazoans to plants, including members such as haemocyanin, polyphenol oxidase, catechol oxidase, PPO and tryosinase. Phylogenetic analysis has revealed that PPO is closely associated with arthropod haemocyanins (van Holde *et al*., 2001). Haemocyanin can acquire PO activity in the presence of sodium dodecyl sulphate (SDS) at low concentrations. Similarly, certain chemicals, such as ethanol or CPC, can activate rPPO3 in the presence of Cu^{2+} (Fig. 2B), and *Of*SP105 can also cleaved and activated by rPPO3. These results confirm that our rPPO3 possesses enzyme activity. A previous study showed that serine proteinase homologues (SPHs) were critical for producing active PO (Gupta *et al*., 2005).

A prominent feature of the innate immune system in insects is the activation of serine proteinase cascade pathways in the haemolymph, which trigger plasma proteins to perform immune functions (Kanost & Jiang, 2015). The PPO activation pathway, like the complementary system in vertebrates, is a proteolytic cascade containing several SPs and their inhibitors. PPO is converted to active PO by PPO activating protease (PAP), and in the haemolymph, PAP is also present as an inactive enzyme, requiring another SP for its activation (Wang & Jiang, 2007). Based on biochemical analyses, PPO is thought to be activated by cleavage at Arg^{51} of the N-terminus in *M. sexta*, *Bo. mori* and *Holotrichia diomphalia* (Jiang *et al*., 1998; Satoh *et al*., 1999; Kim *et al*., 2002). In the *Pacifastacus leniusculus*, the cleavage site of PPO is Arg^{176} (Aspan *et al*., 1995). In *H. diomphalia*, however, there is a second cleavage site at Arg^{162} (Kim *et al*., 2002).

In our study, we showed that proteolytic activation of rPPO3 can be accomplished by immune-challenged mosquito haemolymph and *O. furnacalis* SP105. Moreover, PPO3 can be cleaved *in vivo* in mosquitoes infected with *Be. bassiana*. The immunoblot analysis indicated that rPPO3 can be cleaved into three bands of approximately 60, 50 and 18 kDa (Fig. 3A). Moreover, we found that there was an obvious band around 50 kDa following PPO3 cleavage (Figs 3, 4). Given the size of the cleaved protein fragments, we speculate that the 60 kDa band is cleaved again at Arg^{276}. In arthropods, CLIPs are involved in many biological processes including immune responses and embryonic development. PPO activation requires a protease with trypsin-like specificity to cleave at a specific Arg or Lys residue. Several proteases such as CLIPB8 and B9 have been reported to be involved in activation of melanization (Zou *et al*., 2010; Zhang *et al*., 2016).

Previous studies found that PPO was proteolytically activated and participated in the direct killing of bacteria in *M. sexta* (Soderhall & Ajaxon, 1982; Zhao *et al*., 2007). In our study, we found that activated rPPO3 can kill fungi conidia in the presence of dopamine *in vitro* (Fig. 5D). PO catalyzes dopamine to generate quinones, which finally form melanin and deposit on the cell surface of bacteria and kill them (Zhao *et al*., 2007). Compared with the control groups, only a few of the conidia expressing GFP were detected in the presence of rPPO3 and dopamine. At the same time, a number of conidia without fluorescence were detected.

Several reports have revealed the immune mechanism involved in the interaction between entomopathogenic fungi and host insects. In *An. gambiae* injected with

fungus, a melanin tumour eventually formed around conidia, germ tubes and hyphae (Yassine *et al.*, 2012). Fungal infection affected the longevity of *Ae. aegypti* but restricted the transmission of dengue virus via activated Toll and Janus kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathways (Dong *et al.*, 2012). In our study, rPPO3 was activated *in vitro* by distinct factors and PO3 was also found to catalyse dopamine to kill fungal conidia. Additionally, in our survival experiments mosquitoes were more sensitive to fungal infection after treatment with PPO3 dsRNA, whereas purified rPPO3 increased the survival rate of mosquitoes upon fungal infection (Fig. 7). These experiments have clearly indicated that activated PPO3 is required for survival following fungal infection in *Ae. aegypti*.

Ae. aegypti PPO genes are members of a multigene family involved in melanization and studies on PPO activation in the mosquito immune response are still limited. A model of the melanization pathway in *Ae. aegypti* in response to microbial infection is shown in Fig. S4. PPO3 was cleaved in the haemolymph of fungal-challenged mosquitoes, and the reaction was regulated by CLSP2 (Wang *et al.*, 2015). In future studies, we want to confirm whether IMP-1, IMP-2 or other SPs directly cleave PPO3, and identify the SPH involved in the activation of PPO3. Our present results further our knowledge of the biochemical properties and functions of PPO3 both *in vitro* and *in vivo*. Overall, our study has demonstrated that PPO3 is involved in the antifungal immune response and this contributes to a better understanding of the mechanism of melanization in mosquitoes.

Experimental procedures

Experimental animals and immune challenge

The *Ae. aegypti* UGAL strain was maintained in the laboratory as described previously (Chu *et al.*, 2017). Adult mosquitoes were continuously fed water and 10% sucrose solution. To initiate egg development, mosquitoes were blood fed on chickens. All procedures for using vertebrate animals were approved by the Institute of Zoology Animal Care and Use Committee (Beijing, China). Septic injury by *Be. bassiana* (ARSEF2860) or *En. cloacae* was performed as described previously (Wang *et al.*, 2015).

Expression and purification of rPPO3 and immunoblot analysis

To produce *Ae. aegypti* rPPO3 (*Aa*rPPO3), PPO3 (AAEL011763) cDNA was amplified by PCR using specific primers (Table S2), and cloned into pET28a with 6 × His-tag at both the N- and C-terminus, and transformed into *Es. coli* strain BL21.

Bacteria in 500 ml culture were harvested and re-suspended in 24 ml lysis buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.5). After sonication and centrifugation, the supernatant was transferred to a new tube and spun at 4500 *g* for 15 min at 4 °C after adding 18 ml saturated ammonium sulphate to a final concentration of 35%; the resulting precipitate was suspended in the lysis buffer and added to the column and gently incubated with Ni-NTA beads. Recombinant protein was eluted with elution buffer and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Aliquots of rPPO3 were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with the primary antibodies against the His tag (1:3000; CW0286A) or PPO3 (1:8000; prepared by Beijing Protein Innovation, Beijing China) overnight at 4 °C, respectively (Wang *et al.*, 2015). Immune complexes were visualized by SuperSignal West Pico Substrate (Pierce, Rockford, IL, USA) and developed using X-ray films.

rPPO3 activity assay

To identify rPPO3 via native gel electrophoresis, 30 μl rPPO3 (0.1 μg/μl) with or without Cu^{2+} was mixed with nondenatured gel sample loading buffer (CWBIO, Beijing, China) for 5 min, and separated on gel at 4 °C. The gel was first incubated with 10 mM dopamine dissolved in 10 mM phosphate buffer (pH 6.5) for 10 min and developed in 30% ethanol until protein bands representing rPPO3 clearly appeared (Liu *et al.*, 2012).

We activated rPPO3 in different ways and assayed its activity. Briefly, 20 μl rPPO3 was incubated with 0.5 mM Cu^{2+} for 5 min at room temperature. Then, 1 μl fungal-challenged mosquito haemolymph, 30% final concentration of ethanol, or 10 μl CPC (1 ng/μl) was added and incubated for an additional 10 min. Following this, 200 μl of 0.1 mM dopamine was added and incubated for 10 min, and the absorbance at 470 nm was measured using a microplate reader. One unit (U) of PO activity was defined as an increase of 0.001 absorbance in 1 min (Jiang *et al.*, 2003). Error bars represent the means ± SD from three replicates. Figures were generated using GraphPad 6.0 software (California, USA).

Immunoblot analysis of activated rPPO3

To analyse activation of rPPO3 by fungal-challenged mosquito haemolymph *in vitro*, approximately 1.0 μg rPPO3 was incubated with 1 μl haemolymph from fungal-challenged mosquitoes and lysis buffer in a total volume of 10 μl at room temperature for 60 min. The control group was treated with 1 μl 1 × protease inhibitor (Roche, Indianapolis, IN, USA). Samples were separated by SDS-PAGE and analysed by immunoblot analysis using anti-His antibodies.

To assess rPPO3 activation by SP *in vitro*, SP105 from *O. furnacalis* (*Of*SP105) was used. Purified recombinant $proSP105_{Xa}$ was activated as described previously (Chu *et al.*, 2017). rPPO3 (1.0 μg) was added to the mixture and allowed to react at room temperature for 1 h. *D. melanogaster* rPPO1 (*Dm*rPPO1) activated by SP105 and *Aa*rPPO3 activated by fungal-challenged haemolymph were used as positive controls. The samples were separated by SDS-PAGE and analysed by immunoblot analysis. The activity of *Aa*rPPO3 activated by *Of*SP105 was measured as described above.

LC/Q-TOF-MS and N-terminal determination

To determine the amino acid sequence of the rPPO3 cleaved product, rPPO3 was incubated with fungal-challenged haemolymph and purified using Ni-NTA agarose. The samples were resolved by SDS-PAGE, and bands were excised for subsequent identification using LC/Q-TOF-MS. A second gel was transferred to a PVDF membrane and visualized by Coomassie Blue staining. The target bands (~50 kDa) were excised for N-terminal sequencing, which was performed on the PPSQ-31A (Shimadzu Corporation, Kyoto, Japan) at the Beijing Agriculture Biological Testing Center (Beijing, China).

Tertiary structure prediction

Putative tertiary structure of *Aa*PPO3 was established by the SWISS-MODEL workspace (http://swissmodel.expasy.org/), a web-based tool for 3D structure prediction (Biasini *et al.*, 2014). The experimental structure of *An. gambiae* PPO8 [Protein Data Bank (PDB) accession code 4YZW] was used as a template to build homology models. The quality of the final model was evaluated using the QMEAN Z-score. The representative model chosen for the production of molecular graphics was displayed using PYMOL (http://www.pymol.org/).

Effect of rPPO3 on the growth of Be. bassiana *conidia*

Recombinant PPO3 (0.1 μg/μl, 10 μl) was cleaved by 10 μl activated *Of*SP105. Five μl of fungal conidia (1×10^5/μl) expressing GFP (Bb-GFP) was added to 20 μl rPPO3 activation mixture and dopamine solution. As controls, the conidia were treated with: rPPO3 activation mixture and buffer (50 mM PBS, pH 7.5); dopamine solution and buffer; *Of*SP105 and dopamine solution. After incubation at room temperature for 1.5 h, 10 μl of the reaction and control mixtures was added onto separate glass slides. The fungal conidia were observed using fluorescence confocal microscopy (Zeiss 710, Jena, Germany).

Micro-injection of rPPO3 and survival analysis

About 200 nl rPPO3 was injected into adult female mosquitoes (approximately 0.1 μg/mosquito) infected with *Be. bassiana* or PBS for 24 h via a Nanoliter 2000 injector (World Precision Instrument, Sarasota, Florida, USA). Mosquitoes at 3 days after eclosion were selected for injection. Fifteen minutes after injection, mosquito haemolymph was collected. His-tagged proteins were then purified using Ni-NTA agarose, and samples were analysed by SDS-PAGE and immunoblotting using anti-His antibodies.

Synthesis of dsRNA was accomplished using a T7 RiboMAX Express RNAi kit (Promega, Madison, WI, USA). The *luciferase* gene was used to generate the control iLuc dsRNA. dsRNA was introduced by injection into the thorax of CO_2-anaesthetized female mosquitoes within 1 day posteclosion using the Nanoliter 2000 injector.

To investigate the role of PPO3 in mosquito survival following pathogen invasion, groups of 30 mosquitoes were treated in each experiment. The survival curves were compared using the Kaplan-Meier method, and the *P*-value threshold was calculated with the log-rank or Mantel Cox test (GraphPad 6.0). $P < 0.05$ was considered to be statistically significant.

Acknowledgements

This work was supported by the National Key Plan for Scientific Research and Development of China (nos 2016YFD0500300, 2016YFC1200604), Strategic Priority Research Program of the Chinese Academy of Sciences (nos XDB11030600), the Natural Science Foundation of China (nos 31272367, 31402013, 31672291), Open research Fund Program of State Key Laboratory of Integrated Pest Management (IPM1505, 1609) and National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) Award 5RO1 AI036959.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. (A) The effect of Cu^{2+} on the activity of recombinant prophenoloxidase-3 (rPPO3) activated by ethanol. rPPO3 exhibited no enzyme activity until Cu^{2+} was added and increased as the

concentration of added Cu^{2+} increased. (B) The effect of Cu^{2+} on the activity of purified rPPO3 activated by mosquito haemolymph. Error bars represent means ± SD from three replicates. Different lowercase letters (a–f) represent significant differences amongst the different concentrations of Cu^{2+}.

Figure S2. Predicted tertiary structures of *Ae. aegypti* prophenoloxidase-3. Overall structure of the homodimer with each subunit was shown in cyan and green.

Figure S3. Immunoblot analysis of haemolymph prophenoloxidase-3 (PPO3) in mosquitoes treated with PPO3 double-stranded RNA (dsRNA). iLuc, luciferase dsRNA treated mosquitoes; iPPO3, PPO3 dsRNA treated mosquitoes. PPO3 antibodies were used for the immunoblot analysis. *Aedes aegypti* lipophorin II was used as loading control.

Figure S4. A proposed model of melanization pathway in *Aedes aegypti* in response to microbial infection. Possible involvement of serine proteases (SPs) in activation of prophenoloxidase-3 is shown by broken lines. CLSP2 and serpin-1 negatively regulate this pathway.

Table S1. *Aedes aegypti* proteins detected in the high mass complex by liquid chromatography/quadrupole time-of-flight mass spectrometry.

Table S2. Primers used for protein expression and double-stranded RNA synthesis.