Activity of Fusion Prophenoloxidase-GFP and Its Potential Applications for Innate Immunity Study

Bing Yang1, Anrui Lu1, Qin Peng1, Qing-Zhi Ling2, Erjun Ling1*

1 Key Laboratory of Insect Developmental and Evolutionary Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, People’s Republic of China, 2 Department of Applied Biology, Zhejiang Pharmaceutical College, Ningbo, People’s Republic of China

Abstract

Insect prophenoloxidase (PPO) has been studied for over a century [1]. PPO is a copper-containing enzyme that oxidizes mono-phenols to produce melamins [1,2]. This family of proteins exist in microbes, plants, invertebrate and vertebrate animals [3–7], and each of them has some specific functions. Insect PPO is a very important innate immunity protein that can induce melanization of microbes and parasites thereby quickly and effectively eliminating them [1,3,8–10]. In addition to inducing melanization on the surface of invading pathogens [19,24], besides inducing melanization on the surface of invading pathogens [8,25], PPO is also responsible for melanization at wounds to prevent further infection [26]. To important disease vectors, PPOs of mosquitoes have been extensively studied and shown to be involved in the anti-Plasmodium and anti-bacterial response in both the midgut and the hemocoel [25,27–31]. Drosophila melanogaster, as a powerful genetic tool, is an invaluable model for studying PPO activation and regulation [32]. Thus, work done in small dipteran insects has greatly increased our understanding of PPO activation [27,28,32].

Insect PPO is an important component of humoral and cellular immunity [3,19–21]. Based on the pioneering studies by several research teams [3,19–21], the biochemical pathways leading to PPO activation and melanization are well understood. Thezymogen PPO must be cleaved at a conserved site by terminal serine proteases, and this occurs upon the recognition of polysaccharides on the surface of microorganisms by pattern recognition proteins such as PGRP, βGRP and C-type lectins [3,19–21]. Moreover, the terminal serine proteases also need cleaving to become activated, and serine protease inhibitors (serpins) negatively regulate this process [22,23]. For example, in M. sexta, two terminal serine proteases, PAP2 and PAP3, become activated to cleave plasma PPO with the help of serine protease homologues (SPH), and this is negatively regulated by serpins [19,24]. Besides inducing melanization on the surface of invading pathogens [8,25], PPO is also responsible for melanization at wounds to prevent further infection [26]. To important disease vectors, PPOs of mosquitoes have been extensively studied and shown to be involved in the anti-Plasmodium and anti-bacterial response in both the midgut and the hemocoel [25,27–31]. Drosophila melanogaster, as a powerful genetic tool, is an invaluable model for studying PPO activation and regulation [32]. Thus, work done in small dipteran insects has greatly increased our understanding of PPO activation [27,28,32].

Recently, discoveries of insect PPO function have expanded our knowledge on the regulation of the PPO pathway. Originally, PPO activation was thought to be an independent pathway. However, work on Tribolium castaneum shows that there is crosstalk
between PPO activation and the Toll pathway [33], which presumably allows the insect to respond to infection more rapidly and effectively. *Drosophila* PPO was recently identified as an important component of the clotting system, and to be responsible for sclerotization and melanization around wounds [12,34]. *Drosophila* has three PPO genes, PPO1 (CG5779), PPO2 (CG5193) and PPO3 (CG2952). PPO1 and PPO2 are produced by crystal cells [35,36], However PPO3 can be expressed in lamellocytes when *Drosophila* is infected by parasites [37]. In each species of mosquito, there is up to 10 PPO genes [8–10,28], but there are normally 2–3 PPO genes in other species of insects [1,3,21]. We have no idea why mosquito needs so many PPO genes. Although PPO is a very important factor to induce the melanization of malaria parasites, we know very little of the role of each PPO in the process of melanization. Therefore, it is very important to identify their functions separately in the mosquito which might be helpful to fight against malaria transmission by mosquito. For this purpose, using *Drosophila* PPO genes as a model, the PPOs were recently over-expressed in eukaryotic and prokaryotic cells for identifying their biochemical properties [36,38]. An important finding is that *Drosophila* PPO can be expressed even if there is not enough Cu²⁺ in the culture medium. The apo-rPPO (inactive) becomes holo-PPO (active) in the presence of Cu²⁺, which makes it convenient to express enough rPPO for purification [36,38]. When the three rPPOs were over-expressed in S2 cells respectively, rPPO1 and rPPO2 needed additional Cu²⁺ to achieve a status that permits activation for subsequent L-DOPA or dopamine staining. However, rPPO3 did not need additional Cu²⁺ to become active [36]. A very interesting phenomenon is that when additional Cu²⁺ or substrate was added to the cells, S2 cells with rPPO3 over-expressed became auto-melanized. No cleavage of rPPO3 was discovered [36]. When expressed in different tissues of transgenic *Drosophila*, PPO3 can also induce auto-melanization in those tissues [39]. Further research shows that specific amino acids around the active site pocket affect each rPPO activity by influencing the pocket size [40]. rPPO3 has the largest pocket, and thus, substrate can enter the enzyme to cause auto-melanization even without being cleaved by serine protease, which is a kind of enzyme activity leakage [40].

In the field of insect PPO study, many interesting questions remain unanswered [1]. For example, how is PPO released from hemocytes into plasma? Since PPO has no signal peptide, it is probably released from hemocytes after cell lysis [3], which is also under control by some proteins and chemicals [11,41]. If PPO could be tagged with a fluorescent protein like GFP, this and other questions could be more clearly answered.

In this study, we independently expressed in S2 cells three *Drosophila* PPOs after fusing each of them with GFP at the C-terminus (rPPO-GFP). Our results show that each rPPO-GFP has similar properties as the corresponding unmodified rPPO. Furthermore, purified rPPO1-GFP can be cleaved and activated by *Drosophila* serine proteases to become an active PO. Just like silkworm plasma PPO, rPPO1-GFP can also bind microorganisms after being mixed with silkworm plasma. These data suggest that in vivo expression of rPPO-GFP could be used for the study of immune processes involving the phenoloxidase pathway.

**Results**

**Activities of Fusion Prophenoloxidase-GFP Expressed in S2 Cells**

Three *Drosophila melanogaster* prophenoloxidase (PPO) cDNAs with GFP fused at the C-terminal (rPPO-GFP) were sub-cloned in S2 cells and over-expressed. When rPPO1, rPPO2 and rPPO3 were over-expressed, they exhibited different biochemical properties [36]. If there is not enough Cu²⁺ in the culture medium, rPPO1 and rPPO2 have no activity. Some of the rPPO3 does have activity immediately after being expressed (Table 1). The enzyme activities and fluorescence properties of each rPPO and rPPO1-GFP, rPPO2-GFP and rPPO3-GFP were studied and compared. rPPO1-GFP expressed in S2 cells was first identified by LC-MS/MS, and peptides from PPO1 and GFP were observed (Fig. S1, Table S2), which indicates that the fusion rPPO1-GFP can be expressed in S2 cells. When Cu²⁺ was not added during transfection, many cells had green fluorescence, indicating the expression of rPPO1-GFP (Fig. 1A–1C). However, no cells were stained, indicating no enzyme activity (Fig. 1C). If Cu²⁺ was added, S2 cells with GFP fluorescence became melanized after staining for enzyme activity (Fig. 1D–1E), indicating that rPPO1-GFP had enzyme activity after the addition of Cu²⁺ and activation by ethanol. Enzyme activities of rPPO1 and rPPO1-GFP were then compared. Without Cu²⁺ added during transfection, S2 cells with either rPPO1 or rPPO1-GFP did not stain for enzyme activity (Fig. 1G and 1H). If additional Cu²⁺ was provided, S2 cells with rPPO1 or rPPO1-GFP expressed were strongly stained for enzyme activity (Fig. 1I and 1J). These data corroborate that Cu²⁺ is necessary for apo-rPPO1 and apo-rPPO1-GFP to become holo-rPPO1 and holo-rPPO1-GFP. Finally, the enzyme activity of rPPO1-GFP was significantly lower than that of rPPO1 if Cu²⁺ was added (Fig. 1K), but the expression of rPPO1-GFP and rPPO1 was independent of Cu²⁺ addition during cell transfection (Fig. 1L).

For rPPO2-GFP, when Cu²⁺ was not added during transfection, S2 cells with green fluorescence did not stain for enzyme activity (Fig. 2A–2C), but they were positively stained if Cu²⁺ was added (Fig. 2D–2F). To compare rPPO2-GFP and rPPO2, fluorescence assays showed that, in the absence of Cu²⁺, only rPPO2-GFP had green fluorescence. But when Cu²⁺ was added, both rPPO2-GFP and rPPO2 had PO activity after staining (Fig. 2G–2J). The activity of rPPO2-GFP in cell lysates is the same as that of rPPO2 when Cu²⁺ was added during cell transfection (Fig. 2K). Cu²⁺ had no influence on either rPPO2 or rPPO2-GFP expression in S2 cells (Fig. 2L), but was required for PO activation induced by ethanol.

Similar analyses showed that rPPO3-GFP enzymatic activity was comparable to that of rPPO3. If Cu²⁺ was not added during cell transfection, many cells that express rPPO3-GFP, as indicated by green fluorescence, were stained black (Fig. 3A–3C). However, in the absence of Cu²⁺, some cells stained for PO activity, and some other cells with fluorescence had no PO activity after staining (Fig. 3A–3C). When Cu²⁺ was added during cell transfection, many S2 cells became auto-melanized and had no fluorescence, likely due to fluorescence quenching by melanin (Fig. 3D–3E). However, all cells with visible green fluorescence were stained black after direct addition of dopamine (Fig. 3D–3E). When rPPO3 was compared to rPPO3-GFP, cell staining was similar when Cu²⁺ was not added (Fig. 3G–3H). Some cells with green fluorescence lacked PO activity when rPPO3-GFP was expressed (Fig. 3G), and this is likely due to the presence of newly produced rPPO3-GFP, but the absence of sufficient Cu²⁺ to complete the conformation change of the enzyme. But if Cu²⁺ was added, all cells expressing rPPO3 or rPPO3-GFP were positively stained for PO activity (Fig. 3I and 3J). Many S2 cells with rPPO3 or rPPO3-GFP expressed were stained black even if Cu²⁺ was not added, which is different from rPPO1, rPPO1-GFP, rPPO2 and rPPO2-GFP. Furthermore, rPPO3 and rPPO3-GFP activity was similar when Cu²⁺ was added (Fig. 3K). However, rPPO3-GFP had significantly lower enzyme activities when Cu²⁺ was not added.
which is the same as rPPO3. Finally, PPO3 could not be efficiently detected when Cu\(^{2+}\) was added, suggesting that this enzyme may form large complexes with other proteins during the progress of auto-melanization, and that these complexes cannot be dissociated during Western blot analyses (Fig. 3L). When phenylthiourea (PTU) was added with Cu\(^{2+}\) to inhibit auto-melanization, the protein levels were the same as the group without Cu\(^{2+}\) added (data not shown).

The above experiments show that each rPPO-GFP has almost similar properties as the corresponding wild type rPPO, although there might be some differences in their enzymatic activities. The information is summarized in Table 1.

### Table 1. Summary of the biochemical properties of each rPPO-GFP and the corresponding rPPO.

|                | PPO1 | PPO1-GFP | PPO2 | PPO2-GFP | PPO3 | PPO3-GFP |
|----------------|------|----------|------|----------|------|----------|
| Cell staining  | +Cu\(^{2+}\) | Yes | Yes | Yes | Yes | Yes |
|               | −Cu\(^{2+}\) | No | No | No | No | Yes (Some cells) |
| Auto-melanization | +Cu\(^{2+}\) | No | No | No | No | Yes |
|               | −Cu\(^{2+}\) | No | No | No | No | Yes |
| PO activity   | +Cu\(^{2+}\) | Higher | Lower | Equal | Equal | Equal |
|               | −Cu\(^{2+}\) | No | No | No | No | Yes |
| Protein expression | +Cu\(^{2+}\) | Yes | Yes | Yes | Yes | Yes |
|               | −Cu\(^{2+}\) | Yes | Yes | Yes | Yes | Yes |

Figure 1. Phenoloxidase in S2 cells over-expressing rPPO1-GFP. DIC and fluorescence images were taken in the absence (A–C; −Cu\(^{2+}\)) or presence (D–F; +Cu\(^{2+}\)) of Cu\(^{2+}\) during cell transfection. In both cases, cells with green fluorescence were detected (A, B, D, E), and in the presence of Cu\(^{2+}\) those fluorescent cells displayed PO activity (dark brown) when incubated with dopamine dissolved in 30% ethanol (F). (G-J). Comparison of cells with rPPO1 and rPPO1-GFP expressed. rPPO1 and rPPO1-GFP were over-expressed in S2 cells in the absence (G, H) or presence (I, J) of Cu\(^{2+}\) during cell transfection. The cells were then stained for PO activity. Images represent DIC and fluorescence overlays. When Cu\(^{2+}\) was not added, no cells stained for PO activity (G, H) even though some cells expressed rPPO1-GFP (green fluorescence). When Cu\(^{2+}\) was added, many cells stained for PO activity (I, J; brown pigment), and no fluorescent cells were observed after PO staining due to quenching by melanin. (K) Comparison of rPPO1 and rPPO1-GFP enzyme activities. The amounts of rPPO1 and PPO1-GFP in S2 cell lysates were normalized and determined using purified rPPO1 as a standard by Western blot. Ethanol was used for enzyme activation. When Cu\(^{2+}\) was added, rPPO1 had significantly higher enzyme activity than rPPO1-GFP. No enzyme activities were detected if Cu\(^{2+}\) was not added, which is in agreement with the cell staining shown in (G-J). Columns represent the mean of individual measurements ± S.E.M (n = 3). Significant differences were calculated using the unpaired t test. (L) A Western blot showed that rPPO1-GFP and rPPO1 protein expression occurred regardless of the presence or absence of Cu\(^{2+}\). Bar: 20 μm.

doi:10.1371/journal.pone.0064106.t001
rPPO1-GFP/rPPO2-DsRed or rPPO3-GFP/rPPO2-DsRed were co-transfected into S2 cells without Cu²⁺ added. Forty-eight hours later, S2 cells were observed and counted. For each trial, we always observed that there were S2 cells with green fluorescence, red fluorescence, or with green and red fluorescence at the same time (yellow fluorescence after merging; Fig. 4B, 4E). Green fluorescence indicates the expression of rPPO1-GFP or rPPO3-GFP alone. Red fluorescence indicates the expression of rPPO2-DsRed. Yellow fluorescence means that either rPPO1-GFP or rPPO3-GFP was co-expressed with rPPO2-DsRed in the same cells. While the majority of cells expressed both transfected PPOs, statistical evaluation as shown in Fig. 4C and 4F indicates that plasmids containing different rPPO genes are not always over-expressed in the same cells.

**Activation of rPPO-GFP by Serine Protease**

Cells expressing rPPO-GFP or rPPO-DsRed are easily detected using fluorescence microscopes, and the PPO enzyme can be activated by ethanol (Fig. 1K, 2K, 3K). With this knowledge, we sought to determine whether rPPO-GFP can be cleaved and activated by serine proteases, and this was done using rPPO1-GFP purified from transformed E. coli. These transfected bacteria cells exhibit green fluorescence, and the PPO enzyme can be activated using ethanol and AMM1 respectively, and they all had enzyme activity (Fig. 5D). rPPO1 and rPPO1-GFP were cleaved by serine proteases, leading to the conversion to rPPO1 and rPPO1-GFP (Fig. 5E). Therefore, the GFP-tag does not disturb serine protease cleavage of fusion proteins.

**rPPO1-GFP Binds to Bacteria and Fungi**

M. sexta PPO is known to bind to gram-positive bacteria like Bacillus thuringiensis and Staphylococcus aureus and gram-negative bacteria like Serratia marcescens but not E. coli [42]. In order to determine whether rPPO1-GFP can be used for innate immunity studies, purified rPPO1-GFP was mixed with plasma from silkworm larvae. Approximately 1×10⁹ E. coli, or 1 mg dried Micrococcus, or 1 mg wet Beauveria bassiana spores (Bb. Spore) were mixed with 5 µl plasma containing rPPO1-GFP (5 µg) in a 200 µl incubation system. Western blot assays show that rPPO1-GFP is present in the eluted solution when Micrococcus and Beauveria bassiana spores were used for the binding assay (Fig. 6A). There was also degraded rPPO1-GFP found in the eluted solution when E. coli was used for a pull-down. Moreover, when antibody against Bombyx mori PPO was used, silkworm PPO was also able to bind to Micrococcus and Beauveria spores but not to E. coli (Fig. 6B). In the silkworm plasma, a smaller band was detected by PPO antibody. However, this band is smaller than rPPO1 (data not shown), indicating that it is not activated phenoloxidase. Purified rGFP did not bind to any of the microbes assayed (Fig. 6C).

**Discussion**

PPO has many important physiological functions, such as the melanization of invading pathogens and parasites, wound healing and cuticle sclerotization [3]. Insect PPO belongs to type 3 copper-
Figure 3. Phenoloxidase in S2 cells over-expressing rPPO3-GFP. DIC and fluorescence images were taken in the absence (A–C; –Cu²⁺) or presence (D–F; +Cu²⁺) of Cu²⁺ during cell transfection. At 48 h, cells with green fluorescence were detected (A, B, D, E), and samples were then stained for PO activity (C, F) as described in Figure 1A–F. When Cu²⁺ was absent, some cells with green fluorescence (B) were stained dark brown (C), which is different from rPO1-GFP (Figure 1) and rPO2-GFP (Figure 2). There were also cells with green fluorescence that were not stained for PO activity (C). When Cu²⁺ was present, cells auto-melanized (see arrow in D). The arrowheads point to cells (E) with green fluorescence that were positively stained. (G–J) rPPO3 and rPPO3-GFP were over-expressed in S2 cells in the absence (G, H) or presence (I, J) of Cu²⁺ for 48 h for comparison. In the absence of Cu²⁺, there were still many cells with PO activity (G, H), and many cells were also fluorescent but without PO activity (G). When Cu²⁺ was present during transfection, many cells expressing rPPO3 or rPPO3-GFP were stained dark brown (I, J), and some of these cells were auto-melanized as shown in (D). (K) rPO3 activity assay as shown in Figure 1K. When Cu²⁺ was present, rPPO3 had almost the same enzyme activity as rPPO3-GFP, and these levels were significantly higher than when Cu²⁺ was not added during transfection. Columns represent the mean of individual measurements ± S.E.M (n = 3). Significant differences were calculated using the unpaired t test. (L) Western blot showing that rPPO3-GFP and rPPO3 protein expression occurred regardless of the presence or absence of Cu²⁺. However, due to auto-melanization, in the presence of Cu²⁺ some rPPO3 and rPPO3-GFP form large complexes that were not easily separated by SDS-PAGE. Thus, the amounts of rPPO3 and rPPO3-GFP seem lower than those without Cu²⁺ added. Bar: 20 μm.

doii:10.1371/journal.pone.0064106.g003

containing proteins that extensively exist in mammals, insects, shrimp, plants and microbes [3–7]. The biochemical properties of insect PPOs are conserved. Although we have a good understanding of insect PPO activation, many questions remain [1]. One of them is that PPO is thought to be released through hemocytes rupturing in the hemocoel [3]. However, PPO is produced in the silkworm hindgut and is continuously released into the hindgut lumen for clearance of microbe flora through the melanization of feces [16]. Obviously, PPO released from hindgut cells is different from that released from hemocytes since there is no proof that hindgut cells lyse during release. If we could add a tag like GFP to PPO, it should be easier to determine how PPO is released into the hemolymph or to trace its movement when the insect hosts are wounded or infected.

In this study, GFP was fused at the C-termini of three different Drosophila PPOs. In addition, DsRed was fused with Drosophila rPO2 for a co-expression assay. Fluorescence and enzymatic assays showed that when Cu²⁺ was added, S2 cells that had fluorescence also had PO activity when ethanol was used for activation (Fig. 1, 2, 3), and that each PPO-GFP possessed similar biochemical properties as the corresponding non-GFP tagged rPPO (Table 1), just as rPO1, rPO1-GFP could be activated by serine proteases to show rPO1 activity (Fig. 5D), which indicates that the GFP-tag has no influence on PPO activation. rPO1 activity in S2 cells is lower than that in E. coli after expression (Fig. 1 and Fig. 5). The exact reason is unclear. We conclude that rPO1 expressed in S2 cells in S2 cells may be modified more or less to loss some activity, which needs further studying in the future. The crystal structure of M. sexta PPO indicates that PPO1 and PPO2 can form heterodimer in a back-to-back mode [43,44]. We have no idea whether the heterodimers can be broken during the process of activation after being cleaved by serine proteases. However, GFP fused at the C-terminus did not break down the structure of PPO since each rPPO-GFP in cell lysates had enzyme activity (Fig. 1–3). The tagged GFP can decrease rPO1 activity (Fig. 1K). Therefore, a dimer structure or grafting a different protein at C-terminus may affect the entrance of substrate into the active site pockets of rPO1. However, the tagged GFP at the C-terminus has no obvious effect on rPO2 activity or rPO3 activity (Fig. 2K and 3K). We conclude that there might be some unknown factors in S2 cells that probably affect the activated rPO1-GFP but not rPO2-GFP or rPO3-GFP activities. Obviously, GFP does not interrupt the progress of PPO activation induced either by ethanol or serine protease but it may affect enzyme activities. It is a new different biochemical property among three Drosophila PPOs since they can be fused with GFP-tag and still have enzyme activities. When rPO1-GFP was expressed inside S2 cells, it cannot be induced into secretion into medium with or without Micrococcus or
LTA in vitro (Fig. S2). We believe rPPO-GFP may be helpful to trace PPO released from crystal cells in vivo if we could construct a transgenic fly to over-express those proteins in hemocytes.

In the previous work, we found that the transfection efficiency for the three rPPO genes is different [36]. It seems that rPPO3 has the highest transfection efficiency, rPPO1 is medium, and rPPO2 is the lowest. GFP was used to monitored cell transfection efficiency so as to maintain the working conditions as the same each time. When rPPO2-DsRed was co-expressed with rPPO1-GFP or rPPO3-GFP, cells with both green and red fluorescence had rPPO2-DsRed and rPPO1-GFP or rPPO3-GFP co-expressed at the same time. However, the data show that there are always some cells with green or red fluorescence alone, which indicates that there are some S2 cells that are not suitable for specific rPPO expression. In those cells the transfected plasmid might be degraded or protein translation is inhibited by an unknown mechanism. In addition, the S2 cell is a type of hemocyte cell line [45]. Since PPO1 and PPO2 can only be expressed in crystal cells [35], and PPO3 is expressed in lamellocytes [37], this may partly explain different expression of three Drosophila PPO genes in S2 cells in this work and a previous paper [36].

In vivo, plasma PPO can bind bacteria to initiate immune responses [8,25,42]. Here, when rPPO1-GFP was mixed with silkworm plasma for a bacteria or Beauveria spores binding assay, rPPO1-GFP was observed to bind to Micrococcus cells and fungal spores (Fig. 6A). When antiserum against the silkworm PPO was used, silkworm PPO was clearly observed to bind Micrococcus cells and Beauveria spores (Fig. 6B). Therefore, rPPO-GFP is involved in the recognition of pathogens. In addition, rPPO1-GFP still has PO activity and induces melanization after being activated and provided substrates. Together, the above work indicates that if rPPO-GFP could be produced in vivo, it would facilitate studies on the role of PPO in insect innate immune responses.

Materials and Methods

rPPO-GFP and rPPO-DsRed Expression

Three Drosophila melanogaster prophenoloxidase (PPO) genes were separately fused with GFP (for PPO1, PPO2 and PPO3) or DsRed (for PPO2 alone) at the C-terminus using overlapping PCR. The primer pairs are listed in Table S1. The PCR products were subcloned into pAc5.1/V5-HisB (Invitrogen) under the control of the Drosophila actin 5C promoter. Wild type rPPO, rPPO-GFP and rPPO-DsRed were transiently transfected into S2 cells using Effectene™ (Qiagen) as previously described [36]. Copper dichloride (CuCl₂, 500 μM Sigma), if needed for apo-rPPO or apo-rPPO-GFP to become holo-rPPO or holo-rPPO-GFP, was added to the culture medium during transfection [36]. Cells were usually ready for use at 48 h post transfection.

rPPO-GFP Detection in S2 Cells

After being seeded on a glass slide, S2 cells with green fluorescence were detected and imaged using a fluorescence microscope (Olympus BX51) with differential interference contrast optics (DIC) and appropriate fluorescence filters. Insect PPO can be cleaved by a specific serine protease for activation [3,19,21]. This enzyme can also be activated by some detergents and various kinds of alcohol like ethanol, methanol and 2-propanol [3]. We do not know the mechanism of PPO activation induced by ethanol. However, it is a convenient to detect PPO through ethanol

Figure 4. Co-expression of rPPO2-DsRed with either rPPO1-GFP or rPPO3-GFP. Plasmids containing rPPO1-GFP and rPPO2-DsRed (A-B) or rPPO3-GFP and rPPO2-DsRed (D-E) were co-transfected into S2 cells in the absence of Cu²⁺. Cells with green fluorescence (arrows) expressed rPPO1-GFP or rPPO3-GFP, and cells with red fluorescence (asterisks) expressed rPPO2-DsRed. Cells with yellow fluorescence (arrowheads) co-expressed rPPO2-DsRed and either rPPO1-GFP (B) or rPPO3-GFP (E). All images were taken using green and red filters, and were then merged. (C-F) Among PPO positively stained S2 cells, over 60% had yellow fluorescence, approximately 25% had green fluorescence, and less than 10% had red fluorescence (C, F). Obviously, the ratio of cells with yellow fluorescence is significantly higher than that with red or green fluorescence but not all cells were expressing multiple rPPOs at the same time. Columns represent the mean of individual measurements ± S.E.M (n = 4). Significant differences were calculated using the unpaired t test program. Bar: 20 μm.

doi:10.1371/journal.pone.0064106.g004
Enzyme Activity Assay

rPPO-GFP and rPPO were transiently expressed in S2 cells as described for 48 h with or without Cu^{2+} added [36]. S2 cells were collected and suspended in 10 mM Tris buffer (pH 7.4), exposed to three rounds of freezing at −80°C for 2 min, and quickly thawed to room temperature for 4 min. Cells were examined using a microscope to ensure that they had lysed. Some cell lysates, including cell pellets, were used for Western blot assays using each purified rPPO (0.2 μg) as a standard to determine the amount of rPPO or rPPO-GFP expressed in S2 cells. This quantification was performed using ImageJ software (National Institutes of Health).

Purification of rPPO-GFP after Expression in Escherichia coli BL21 (DE3)

The Drosophila three PPO genes can be expressed in Escherichia coli (E. coli) for purifying large amount of rPPO [38]. Although PPO can be expressed inside eukaryotic cells like S2 cells, the production is too low for obtaining large amount of PPO for various biochemical studies. rPPO1-GFP was sub-cloned into pET28a (Invitrogen) for protein expression in E. coli at 16°C as described [38]. E. coli cells with rPPO1-GFP expressed were observed via microscopy to detect GFP fluorescence before protein purification. As a control, rPPO1 was also expressed in E. coli as previously described [38]. rPPO1 and rPPO1-GFP were purified as described but with some modifications [38]. Briefly, bacterial cell pellets were collected after centrifugation and re-suspended in 20 ml Lysis Buffer (10 mM Tris–HCl pH 7.4, 0.2 M NaCl, cells from 250 ml E. coli culture). The above suspension was incubated on ice for 30 min, followed by sonication on ice. After that, the cell lysate was centrifuged at 13,800 g for 30 min at 4°C. Two milliliters of 50% Ni-NTA slurry was equilibrated using 20 ml Buffer (10 mM Tris–HCl pH 7.4, 0.2 M NaCl, cells from 250 ml E. coli culture). The above suspension was incubated on ice for 30 min, followed by sonication on ice. After that, the cell lysate was centrifuged at 13,800 g for 30 min at 4°C. Two milliliters of 50% Ni-NTA slurry was equilibrated using 20 ml Lysis Buffer in a 10 ml empty column. The supernatant was added to the equilibrated column with the bottom outlet capped and mixed gently. The column was placed at 4°C for 60 min to allow protein binding. The column was then washed using Washing Buffer (10 mM Tris, 0.2 M NaCl, 20 mM Imidazole, pH 7.4) until the absorbance at 280 nm was not changed.

Figure 5. rPPO1-GFP can be activated by serine protease. (A, B) rPPO1-GFP was expressed in E. coli cells exhibited green fluorescence (B) as compared with those observed using DIC (A). (C) Purified rPPO1-GFP (0.3 μg) was separated by SDS-PAGE and stained with Coomassie Brilliant Blue, showing that rPPO1-GFP had been purified to homogeneity (left panel in C). rPPO1 (1 μg) and rPPO1-GFP (1 μg) all had enzyme activity in the native gel but their positions were shifted due to the GFP-tag at the C-terminus (right panel in C). (D) Serine proteases activate rPPO1-GFP. A 50 μl system containing purified rPPO1 (3 μg) or rPPO1-GFP (3 μg) and 3 μg AMM1 in 10 mM Tris buffer (pH 8.0) was incubated on ice for 1 h. Ethanol was also used to activate rPPO1 and rPPO1-GFP, and this was done by mixing 3 μg purified protein with an equal volume of 60% ethanol solution and incubating for 5 min. rPPO1 activity is significantly higher than rPPO1-GFP when enzymes were activated either by ethanol or AMM1. Columns represent the mean of individual measurements ± S.E.M (n = 6). Significant differences were calculated using the unpaired t test. (E) Western blot showing that rPPO1 and rPPO1-GFP were cleaved by serine protease in AMM1 (arrowheads). Bar: 20 μm.

doi:10.1371/journal.pone.0064106.g005
rPPO1-GFP was eluted with Elution Buffer (10 mM Tris, 0.2 M NaCl, 100 mM Imidazole, pH 7.4). For each elution fraction, 500 ml solution was collected. The purity of rPPO1 or rPPO1-GFP in each fraction was determined by SDS-PAGE. The fractions containing rPPO1 or rPPO1-GFP with high purity were combined and then the purified rPPO1 or rPPO1-GFP was ultrafiltrated using changing buffer (10 mM Tris, pH 7.4) at 8,000 g at 4 °C for 5 times. Next the concentrations of purified proteins were determined. For long time preservation at 280 °C, 50% glycerol (final concentration) was added and the samples aliquoted. The above purified rPPO1 and rPPO1-GFP then must be added to Cu2+ to achieve a status that can be activated by ethanol or serine proteases [38].

Activation of Purified rPPO1 and rPPO1-GFP by Serine Protease

A protein fraction containing serine proteases from Drosophila melanogaster initially called AMM1 [46], was prepared as described [47,48]. Purified rPPO1 (3 μg) and rPPO1-GFP (3 μg) were then incubated with 3 μg AMM1 in a 50 μl system prepared in 10 mM Tris buffer (pH 8.0) on ice for 1 h. As a comparison, rPPO1 and rPPO1-GFP were also activated using mixing 3 μg purified protein with an equal volume of 60% ethanol solution and incubating for 5 min. The amount equal to original rPPO1 (0.25 μg) was then removed and mixed with 200 μl dopamine (10 mM) dissolved in 10 mM Tris-buffer (pH 7.5), and the absorbance at 490 nm was continuously monitored using an EXPERT 96 microplate reader. To identify rPPO1 and rPPO1-GFP cleavage by AMM1, an amount equal to 0.5 μg protein was used in a Western blot assay.

Microorganisms Preparation and Binding Assay

E. coli was prepared as described [16]. Micrococcus luteus (Sigma) was suspended in sterile 0.85% NaCl. Beauveria bassiana spores were prepared as described [49] and suspended in sterile 0.85% NaCl. Micrococcus and bassiana spores (wet weight) were re-suspended to make a concentration 10 mg/ml.

Insect plasma PPO forms a large complex with other plasma proteins to bind various microorganisms [42]. In order to identify whether rPPO tagged with GFP has such an innate immunity function, 5 μg purified rPPO1-GFP was mixed with 5 μl plasma from naïve Bombyx larvae suspended in a 200 μl system prepared in binding buffer (50 mM Tris buffer, 100 mM NaCl, pH 8.0). To inhibit the auto-melanization of plasma, 5 μl saturated phenylthiourea (PTU) was added and incubated on ice for 30 min. Approximately 1 x 10^9 E. coli cells or 1 mg dried Micrococcus or 1 mg Beauveria bassiana spores were suspended in the above mixture and incubated for 30 min at room temperature as described with modification [42]. The mixtures were centrifuged at 13,000 g for

![Image](image.png)
5 min to pellet the microorganisms and the supernatant was removed. After vortexing and washing with 0.2 ml binding buffer three times, the pellets were suspended in 1× SDS (200 μl) loading buffer and heated to 95 °C for 5 min. The supernatants containing eluted proteins were used in a Western blot assay. To replace rPPO1-GFP, purified rGFP was also used for binding assay as described above.

LC-MS/MS

rPPO1-GFP expressed in S2 cells was identified using a LC-MS/MS assay to ensure that there was no problem with the expression system for different fusion proteins. When rPPO1-GFP was over-expressed in S2 cells for 48 h, cells were then collected and lysed. Approximately 15 μg total proteins were loaded for a denatured SDS-PAGE assay followed by Coomassie Brilliant Blue R-250 staining. Based on Western blot detection, the gel band containing rPPO1-GFP was excised for LC-MS/MS assay as described [16]. The Drosophila protein sequence database used in this analysis was downloaded from UniProt (http://www.uniprot.org/) using the keyword “Drosophila melanogaster” plus green fluorescent protein (GenBank: ADQ43426.1).

S2 Cells Immune Response

rPPO1-GFP was over-expressed in S2 cells for 48 h with 0.5 mM CuCl₂ added in culture medium as described [36]. Then 6 μg Lipoteichoic acid from Bacillus subtilis (LTA) (L2625, Sigma) or 60 μg dried Micrococcus luteus cells was added to S2 cells, respectively. The above cells were collected after 4.5 h incubation. S2 cells were centrifuged and lysed. Approximately 6 μg total proteins or 15 μl medium were loaded per lane for a Western blot assay using a polyclonal antibody against Drosophila rPPO1.

Western Blot Assays

Western blot was performed to detect rPPO or rPPO-GFP expressed in S2 cells as previously described [36]. Rabbit anti-His-tag polyclonal antibody (ABCAM; 1:3, 000) was used to detect each rPPO and rPPO-GFP. Antibodies against Drosophila rPPO1 or against silkworm PPO [50] do not cross react. Therefore, antibodies against Drosophila rPPO1 (1:3, 000), Bombyx PPO (1:5, 000) and rGFP (1:2, 000) were used as the primary antibodies for detecting the corresponding protein in the microorganism binding assay. Goat anti-rabbit IgG conjugated with alkaline phosphatase (AP, Chemicon) (1:5, 000) and rGFP (1:2, 000) were used as the secondary antibody. In order to detect whether rPPO1-GFP can be released into medium from S2 cells, proteins were detected by chemiluminescence catalyzed by a horseradish peroxidase (HRP) conjugated secondary antibody using the Pierce ECL Western Blotting Substrate (32106; Thermo).

Supporting Information

Figure S1 Separation of S2 cell lysate proteins for LC-MS/MS assay. S2 cells transfected with blank vector (as control) or rPPO1-GFP were cultured for 48 h, and then cells were collected and lysed. (A) Protein samples of S2 the control cell lysate (left lane) and the one containing over-expressed rPPO1-GFP (right lane) were separated on SDS-PAGE for Coomassie Brilliant Blue staining. Approximately 15 μg total protein were loaded. (B) Western blot using polyclonal antiserum against rPPO1 to locate rPPO1-GFP position. Another gel was made as shown in (A) for western blot assay. After comparison, the framed area (right lane in A) was excised for LC-MS/MS assay.

Figure S2 rPPO1-GFP is not released into culture medium. rPPO1-GFP was over-expressed in S2 cells for 48 h, and then LTA and Micrococcus were added to those S2 cells respectively for 4.5 h. S2 cells (6 μg) and culture medium (15 μl) were prepared for Western blot assay using polyclonal antibody against Drosophila rPPO1. rPPO1-GFP is not released into culture medium. M: cell culture medium; C: cells with rPPO1-GFP over-expressed.

Table S1 List of primers used for fusing each PPO with GFP or DsRed.

Table S2 List of proteins identified by LC-MS/MS. The framed gel containing rPPO1-GFP as shown in Figure S1 was excised for LC-MS/MS assay. All identified proteins are listed. rPPO1 (GenBank: AAF57775.1) and GFP (GenBank: ADQ43426.1) were labeled in green. Fusion rPPO1-GFP was expressed in S2 cells.

Author Contributions

Conceived and designed the experiments: BY EL. Performed the experiments: BY AL. QP. Analyzed the data: BY EL. Contributed reagents/materials/analysis tools: BY AL. QP. Wrote the paper: QL. EL.
