A New Easter-type Serine Protease Cleaves a Masquerade-like Protein during Prophenoloxidase Activation in Holotrichia diomphalia Larvae

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The prophenoloxidase (proPO) activation pathway, like the vertebrate complement system, consists of a protease cascade and functions as a non-self-recognition system in these animals. Determining the molecular mechanism by which pattern recognition molecules differentiate non-self from self and transduce signals that stimulate defense responses is a key for understanding the ways in which innate immune systems are regulated. However, the proPO system is poorly defined at the molecular level. The proPO-activating system of the insect Holotrichia diomphalia comprises several components, some of which have been cloned and characterized, such as the novel 27-kDa proPO-activating factor-III (PPAF-III) from the plasma of H. diomphalia larvae and two prophenoloxidases. The PPAF-III gene encodes an easter-type serine protease zymogen consisting of 351 amino acid residues with a mass of 40 kDa. The purified 27-kDa PPAF-III specifically cleaved a 55-kDa proPPAF-II to generate a 45-kDa PPAF-II with or without Ca²⁺ present. Furthermore, two Holotrichia prophenoloxidases (proPO-I and -II) have been characterized, and their structural changes during activation were examined in vitro reconstitution experiments. When the proPOs were incubated with PPAF-I, the 79-kDa proPOs were converted to 76-kDa proPOs, which did not exhibit any phenoloxidase (PO) activity. However, when the proPOs were incubated simultaneously with PPAF-I, proPPAF-II, and PPAF-III in the presence of Ca²⁺, a 60-kDa protein (PO-1) with PO activity was detected in addition to the 76-kDa proPO-II protein. These results indicate that the conversion of Holotrichia proPOs to enzymatically active phenoloxidase is accomplished by PPAF-I, PAF-II, and PPAF-III through a two-step limited proteolysis in the presence of Ca²⁺.

The prophenoloxidase (proPO)¹-activating system in invertebrates plays an important role in defense against pathogens and parasites and during cuticular sclerotization. The activation of the proPO system is triggered by elicitors derived from microbial cell walls, such as lipopolysaccharide (LPS), peptidoglycan, and β-1,3-glucan (1–3). Several pattern recognition molecules involved in the proPO system, such as peptidoglycan-binding proteins (4), proteins that bind both LPS and β-1,3-glucan (5, 6), and β-1,3-glucan-binding proteins (7, 8), have been found in various invertebrates. However, the key question is how these pattern recognition molecules can induce activation of the proPO system in response to microbial infection. One hypothesis is that the pattern recognition molecules make a complex with the proPO-activating enzyme(s) and microbial cell wall components, and then activated proPO activating enzyme(s) will convert proPO to active phenoloxidase (PO) by limited proteolysis (1–3).

Recently, we characterized two new proPO-activating factors (PPAF-I and PPAF-II) from the coleopteran Holotrichia diomphalia larvae (9, 10). The overall structure of the 37-kDa proPPAF-I is highly similar to that of Drosophila easter, a serine protease that is essential for pattern formation during normal embryonic development (11). Three other proPO-activating enzymes, from Manduca sexta, Bombyx mori, and Pacificastus leniusculus, have been cloned that are also similar in structure to PPAF-I (12–14). proPPAF-II, with a mass of 55 kDa, is similar to that of Drosophila masquerade (15), a non-proteolytic serine protease-like protein with a mutation in the catalytic triad, making this protein without serine protease activity. This protein is expressed during embryogenesis, larval, and pupal development in Drosophila melanogaster. The proPPAF-II protein contains a trypsin-like serine protease domain in the carboxyl terminus, except for a substitution of Ser in the active site triad to Gly and a clip domain in the amino terminus. Even though the numbers of clip domains are different between proPPAF-I and Drosophila masquerade, the catalytic domain, except for the serine residue and the disulfide linkages, is perfectly conserved in each of these two proteins.

In this study we have investigated the complex activation mechanism of the proPO system in H. diomphalia by isolating and characterizing three new proPO components and by studying the sequential activation of three proPPAFs and two proPOs.

EXPERIMENTAL PROCEDURES

Animals and Collection of Hemolymph—Methods for raising the insects and collecting the hemolymph were as described previously (16). Hemocytes were collected from the hemolymph by centrifugation at 3000 × g for 10 min at 4°C. Hemocytes were washed in 1× Hanks' balanced salt solution (HBSS) and resuspended in HBSS at a density of 2×10⁶ cells/mL. This suspension was used to make the hemocyte preparations for in vitro reconstitution experiments. The hemocytes were then incubated with PPAF-I, PPAF-II, and PPAF-III to examine the effects of these proPO-activating factors on the activation of proPOs.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB079644 (Hd-proPO-I), AB079665 (Hd-proPO-II), and AB079666 (proPPAF-III).

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§ The abbreviations used are: proPO, prophenoloxidase; PO, phenoloxidase; PPAF, prophenoloxidase activating factor; LPS, lipopolysaccharide; FPLC, fast protein liquid chromatography; Hd, Holotrichia diomphalia; Ms, Manduca sexta; Bm, Bombyx mori; Pt, Pacificastus leniusculus; Tt, Tachypleus tridentatus; Dm, Drosophila melanogaster.
200 × g for 10 min at 4 °C, washed with anticoagulation buffer, and stored at −80 °C. About 3 × 10^7 packed cells were obtained from 500 ml of hemolymph. The supernatant was taken as plasma, adjusted to pH 6.5 with 1 N citric acid solution, and stored at −80 °C until use. The hemocyte lysate was prepared according to our previously published method (10). Holotrichia proPOs were purified as described previously (17). The active PPAF-I and proPPAF-II were purified according to our previously published methods (10, 16).

**Assay of PO Activity**—The PO assay was carried out as described previously (9). To examine the effects of purified 27-kDa PPAF-III on proPO activation, a mixture consisting of purified proPOs (2 µg), purified active PPAF-I (1 µg), proPPAF-II (1 µg), and purified PPAF-III protein (1 µg) was added to 400 µl of substrate solution (1 mM 4-methylumbelliferyl 4-hydroxyproline ethyl ester in 20 mM Tris-HCl buffer, pH 8.0) in the presence or absence of 5 mM CaCl2. After incubation at 30 °C for 10 min, the increase in absorbance at 520 nm was measured, and PO activity was expressed as the change in absorbance at 520 nm/10 min of incubation (ΔA_520/10 min).

**Purification of PPAF-III from H. diomphalia Plasma Solution**—After ultracentrifugation of plasma at 203,000 × g for 4 h at 4 °C, the resulting supernatant was diluted 10× with buffer A (50 mM Tris/ HCl (pH 6.5) containing 5 mM EDTA), and about 1000 ml was applied to a dextran sulfate-Sepharose CL-6B column with Ca2 + and 1 M NaCl in buffer A. When the hemocyte lysate containing proPOs and proPPAF-II was incubated with fractions from the dextran sulfate-Sepharose CL-6B column with Ca2 +, fractions showing PO activity were pooled and concentrated by ultrafiltration (YM10, Amicon). We then found that two different fractions were necessary to induce PO activity in the hemocyte lysate; these fractions were named Gr-1 and Gr-2. The Gr-1 fraction contained PPAF-I, which was proven by Western blotting analysis using a PPAF-I antibody. To purify another PPAF from the Gr-2 fraction, namely PPAF-III, 3 ml of the concentrated Gr-2 solution was applied to a Sephacryl S-200 column (52 × 120 cm) equilibrated and eluted with buffer A. Fractions showing PO activity when incubated with hemocyte lysate, Gr-1, and 5 mM Ca2 + were pooled and then were diluted with buffer B (50 mM phosphate, pH 7.0, containing 1.7 M (NH4)2SO4) and loaded onto a phenyl-Sepharose FPLC equilibrated with buffer B. The column was eluted with a linear gradient of 1.7 to 0 M (NH4)2SO4 in buffer B, and fractions containing a 27-kDa PPAF protein were pooled and concentrated by ultrafiltration. The ammonium sulfate was removed by repeated ultrafiltration with buffer C (20 mM Tris/HCl, pH 7.4) and then applied to a Mono-Q FPLC column equilibrated with buffer C. The absorbed proteins were eluted with a linear gradient of 0–0.4 M NaCl in buffer C. Fractions from the Mono-Q FPLC column showing PO activity when incubated with purified proPO, PPAF-I, and proPPAF-II, pooled, and concentrated by ultrafiltration. The purified PPAF-III was reduced, alkylated, and digested with trypsin. Tryptic peptides were separated by HPLC on a C18 reverse-phase column (18). The amino-terminal amino acid sequences of the PPAF-III and internal tryptic peptides were determined using an Applied Biosystems Procise automated gas-phase sequencer (19).

**Characterization of the 27-kDa Protein as a New proPO-activating Factor (PPAF-III)**—We purified proPOs and proPPAF-II from hemocyte lysate and activated PPAF-I and PPAF-III from the plasma of H. diomphalia as shown in Fig. 1A. During dextran sulfate column chromatography, two fractions (Gr-1 and Gr-2) were specifically able to induce PO activity when both fractions and hemocyte lysate were incubated in the presence of Ca2 +. However, PO activity could not be observed when hemocyte lysate was incubated with either Gr-1 or Gr-2 in the presence of Ca2 +. Because the Gr-1 fractions contained activated PPAF-I, as determined by immunoblotting, it was likely that Gr-2 contained another proPO-activating factor(s) necessary for inducing PO activity. Therefore, we purified this factor by pooling Gr-2 fractions and then subjecting these to gel filtration on a Sephacryl S-200 column followed by hydrophobic chromatography using a phenyl-Sepharose column. The active fractions showing PO activity were further purified by Mono-Q FPLC column chromatography. The purified protein ran as a single band under reducing and nonreducing conditions with molecular masses of 27 and 40 kDa, respec-
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**Fig. 2.** Alignment of the amino acid sequence of *H. diomphalia* proPO-I and proPO-II with those of known insect proPOs: *B. mori* proPO-I and -II (*Bm-proPO-I* and *Bm-proPO-II*) (22), *M. sexta* proPO and proPO-I (*Ms-proPO* and *Ms-proPO-I*) (23, 29), *Anopheles gambiae* proPO (Ag-proPO) (30). The numbers refer to the predicted protein sequence. Closed circles indicate the conserved histidine residues of proPOs. Residues conserved in all sequences are shown within boxes. The arrow indicates the conserved cleavage site of proPOs, and the arrowhead indicates the cleavage site of *Holotrichia* proPO-I. Gaps were introduced to obtain maximal sequence similarity.

The mobility pattern is similar to those of several other PPAFs (13, 14) where the mass of the purified PPAF is higher under nonreducing than reducing conditions; this suggests that disulfide bonding is responsible for the higher mass. In total, 56 µg of the purified 27-kDa protein, which we named prophenoloxidase-activating factor III (PPAF-III), was obtained from 33.6 g of plasma. To determine the localization of the 27-kDa PPAF-III, we prepared plasma and hemocyte lysate from *H. diomphalia* larvae and analyzed them by Western blot analysis using an affinity-purified PPAF-III antibody. As shown in Fig. 1C, the purified PPAF-III antibody recognized a protein with an apparent mass of 27 kDa in plasma but not in hemocyte lysate. This result suggests that PPAF-III exists as a zymogen of about 40 kDa in plasma and that the purified 27-kDa PPAF-III is generated by a proteolytic cleavage from the zymogenic proPPAF-III. The six peptides of the PPAF-III in the corresponding open reading frame of 1053 nucleotides corresponding to 351 amino acid residues with a predicted mass of 37,900 Da (Fig. 3). This result suggests that the proPPAF-III exists as a zymogen of 40 kDa in plasma and that the purified 27-kDa PPAF-III is generated by a proteolytic cleavage from the zymogenic 40-kDa proPPAF-III.

Molecular Cloning of *Holotrichia* proPOs and PPAF-III—Both *Holotrichia* proPO-I and proPO-II contain an open reading frame of 2052 nucleotides corresponding to 684 amino acid residues with a predicted mass of 79 kDa. The six chemically determined partial amino acid sequences of proPO-I and proPO-II coincide with the deduced amino acid sequences in the corresponding open reading frame. The amino acid sequence comparisons revealed that proPO-I and proPO-II show high sequence homology to other insect proPOs with highest identity, 60.5 and 72.8%, respectively, to *M. sexta* proPOs. The two copper binding regions containing the six conserved histidine residues of proPO-I and proPO-II are the most conserved regions among all insect proPOs as well as in arthropod hemocyanins (Fig. 2). Both of the *Holotrichia* proPOs contain an Arg–Phe sequence motif around residues 51–52, which is also present in other insect proPOs (indicated by an arrow), and this has been determined to be the cleavage site in both *B. mori* (22) and *M. sexta* (23). Also, proPOs from *H. diomphalia* and *M. sexta* have two other interesting putative cleavage sites for serine proteases (indicated by arrowheads), namely Arg<sup>202</sup>–Ala<sup>203</sup> from *Hd-proPO-I* and *Ms-proPO-I, Gln<sup>163</sup>–Ala<sup>164</sup> motif from *Hd-proPO-II* and Ms-proPO, respectively. However, this motif was not observed in *B. mori* or *Anopheles gambiae* proPO.

To determine the complete amino acid sequence of the purified PPAF-III, we screened the cDNA library of *H. diomphalia* larvae with a degenerate DNA probe. The cDNA of proPPAF-III contains an open reading frame of 1053 nucleotides corresponding to 351 amino acid residues with a predicted mass of 37,900 Da (Fig. 3). This result suggests that the proPPAF-III exists as a zymogen of 40 kDa in plasma and that the purified PPAF-III (27 kDa) is generated by a proteolytic cleavage from the zymogenic proPPAF-III. The six peptides of the PPAF-III in which the amino acid sequences were chemically determined were present in the deduced amino acid sequence. The apparent mass of proPPAF-III on SDS-PAGE under reducing conditions (40 kDa) is slightly larger than the deduced sequence mass (37.9 kDa), which may be explained by the fact that the native protein has undergone some post-translational events. The proPPAF-III has two domains, an amino-terminal domain and a catalytic domain. The hydrophobic first 19 amino acids of the amino-terminal end of the protein probably constitute a signal peptide with a putative cleavage site between Ala<sub>14</sub> and Ile<sub>14</sub> (24). One putative clip motif is present in the amino-terminal domain of the protein (Fig. 3, closed circles). The putative catalytic domain, Val<sup>57</sup>–Val<sup>190</sup>, is characteristic of trypsin-like serine proteases (*arrow*), as is the presence of His<sup>192</sup>, Asp<sup>204</sup>, and Ser<sup>299</sup> residues in the catalytic sites (*stars*). The six-cysteine residues (*diamonds*) that form three disulfide bridges in most serine proteases are present and their positions are also conserved in proPPAF-III. The deduced amino acid sequence of the *Holotrichia* proPPAF-III (Hd-PPAF-III) was similar to *Tachypleus* proclotting enzyme (Tt-PCE, 29.3%) (28),...
Holotrichia PPAF-I (Hd-PPAF-I, 44.5%) (9), M. sexta proPO activating enzyme (Ms-PPAE, 40.6%) (12), B. mori proPO activating enzyme (Bm-PPAE, 34.1%) (13), crayfish P. leniusculus proPO activating enzyme (Pl-PPAE, 35.2%) (14) and D. melanogaster easter (Dm-easter, 36.6%) (11). In conclusion, PPAF-III has a serine protease domain at the carboxyl terminus and a single clip motif in the amino terminus (Fig. 3). However, the biological function of this clip motif has not yet been determined, whereas the clip motif in P. leniusculus PPAE has been demonstrated to act as a defensin-like peptide (14).

Biological Functions of the Purified PPAF-III—When PPAF-III was incubated with the purified Holotrichia proPOs, the active forms of PPAF-I and proPPAF-II, generation of PO activity was shown to be dependent on Ca\(^{2+}\) (column 9 in Fig. 4A) because incubation without Ca\(^{2+}\) failed to induce PO activity (column 8). All other combinations also failed to induce PO activity (Fig. 4A). These results suggest that PPAF-III and Ca\(^{2+}\) are necessary for the triggering of PO activity in the Holotrichia proPO system.

Because purified PPAF-I and PPAF-III have serine protease domains, it is possible that these factors can cleave proPOs and proPPAF-II (a masquerade-like protein without protease activity). We examined several molar ratios of PPAF-III or PPAF-I to the proPOs, such as 0.05:1, 0.1:1, 0.5:1, and 1:1, with constant amount of PPAF-II present (data not shown). When we used small amounts of protease, a longer incubation time was required to induce PO activity. Therefore, to observe PO activity more rapidly, we used excess amounts of proteases.

We first examined the cleavage pattern by incubating these purified factors as shown in Fig. 4B. When PPAF-I and proPPAF-II, or PPAF-I and PPAF-III, were incubated in...
presence of Ca\(^{2+}\), there was no cleavage of proPPAF-II, because neither of these factors had changed their mobility (lanes 4 and 5). In contrast, if proPPAF-II and PPAF-III were incubated with or without Ca\(^{2+}\), the 55-kDa proPPAF-II was cleaved to a 45-kDa PPAF-II, shown as band C in Fig. 4B (lanes 6 and 7). When PPAF-I, proPPAF-II, and PPAF-III were incubated in the presence or absence of Ca\(^{2+}\), proPPAF-II was still the only factor that was cleaved (lanes 8 and 9). To determine the specific cleavage site of the 55-kDa proPPAF-II, the amino-terminal sequence of the generated 45-kDa protein was determined to be ENANEXESYL. This sequence perfectly matches that of Glu\(^{100}\)–Leu\(^{109}\) in PPAF-II, as shown in Fig. 4D-1. This result suggests that PPAF-III can specifically cleave proPPAF-II regardless of whether Ca\(^{2+}\) is present.

Second, we studied the cleavage pattern of the two Holotrichia proPOs and the three PPAFs by performing in vitro reconstitution experiments as shown in Fig. 4C. The two purified Holotrichia proPOs have one band with a mass of 79 kDa on SDS-PAGE under reducing conditions (lane 1). However, when proPOs and PPAF-I were incubated with or without Ca\(^{2+}\), a new 76-kDa protein band (indicated as band A) was generated from the 79-kDa protein, and this 76-kDa protein has no PO activity (lanes 2 and 3). To confirm this cleavage by PPAF-I, we determined the amino-terminal amino acid sequence of the generated 76-kDa protein as shown in Fig. 4D-2. The two resulting amino acid sequences from the 76-kDa band were FGEETKSKIP and FGEDDSETVT, which perfectly matched the Arg\(^{50}\)–Phe\(^{51}\) and Arg\(^{51}\)–Phe\(^{52}\) cleavage sites of Holotrichia proPO-I and proPO-II, respectively. This result shows that purified PPAF-I specifically cleaves the 79-kDa proPO-I and proPO-II into two 76-kDa proteins between the two Arg–Phe motif sites. Interestingly, the two 76-kDa proteins
do not show any PO activity, whereas other insect proPOs, if cleaved by a prophenoloxidase-activating enzyme at Arg-Phe motif, will exhibit PO activity (22, 23). When proPOs were incubated with proPPAF-II and Ca$^{2+}$, PPAF-III with Ca$^{2+}$, or PPAF-III without Ca$^{2+}$, the 76-kDa protein band was not generated from the 79-kDa proPOs, clearly demonstrating that only PPAF I is involved in cleaving the two zymogenic 79-kDa proPOs into the two 76-kDa zymogenic POs. When proPOs, PPAF-I, and proPPAF-II were incubated with Ca$^{2+}$ (lane 7), or when proPOs, PPAF-I and PPAF-III were incubated with Ca$^{2+}$ (lane 8), the 76-kDa protein was generated from 79-kDa proPOs but still without PO activity. In contrast, when proPOs, proPPAF-II, and PPAF-III were incubated without or with Ca$^{2+}$, the 76-kDa band A was not generated from the 79-kDa proPOs. These results suggest that PPAF-II and PPAF-III do not have any effect on the specific cleavage of 79-kDa proPOs. However, proPPAF-II (55 kDa) is specifically cleaved to 76-kDa proPO-I and then this 76-kDa proPO-I was cleaved to 60-kDa and all three factors without and with Ca$^{2+}$ can, or present, the upstream part of this proPO cascade is poorly proPOs in activating enzymes and other factors are necessary to activate these results suggest that PPAF-II and PPAF-III do not have any effect on the specific cleavage of 79-kDa proPOs. However, proPPAF-II (55 kDa) is specifically cleaved to 76-kDa proPO-I and then this 76-kDa proPO-I was cleaved to 60-kDa and all three factors without and with Ca$^{2+}$ can, or present, the upstream part of this proPO cascade is poorly proPOs in activating enzymes and other factors are necessary to activate these results suggest that PPAF-II and PPAF-III do not have any effect on the specific cleavage of 79-kDa proPOs. However, proPPAF-II (55 kDa) is specifically cleaved to 76-kDa proPO-I and then this 76-kDa proPO-I was cleaved to 60-kDa and all three factors without and with Ca$^{2+}$ can, or present, the upstream part of this proPO cascade is poorly proPOs in activating enzymes and other factors are necessary to activate

In this study, we show for the first time that several proPO-activating enzymes and other factors are necessary to activate proPOs in H. diomphalia. Based on these results, we propose a Holotrichia proPO activation cascade as shown in Fig. 5. At present, the upstream part of this proPO cascade is poorly understood. For instance, we do not know how LPS, peptidoglycan, or β-1,3-glucan recognition proteins induce the activation of prophenoloxidase-activating enzyme(s) or other factor(s). The downstream part of the Holotrichia proPO system is quite complex and involves limited proteolysis of several factors and proPOs. An easter-type serine protease, PPAF-I, cleaves the 79-kDa zymogenic proPO-I and -II to two 76-kDa proPO-proteins, still without any PO activity. Then another easter-type serine protease, PPAF-III, cleaves the 55-kDa zymogen proPPAF-II to 45-kDa PPAF-II, and finally the 76-kDa proPO-I generated by PPAF-I is cleaved to an active 60-kDa PO by

Previously, Ashida (26) and Söderhäll (27) and their colleagues published studies on the biochemical characterization of proPO-activating enzymes. Furthermore, they proposed early on a proPO activation cascade in arthropods (for review see Refs. 1-2). Recently, Kanost and colleagues (28) reported that a C-type lectin, M. sexta immunolectin-2, which binds to bacterial LPS, is also involved in proPO activation. It still remains to be elucidated in detail how the connection between the upstream and downstream parts of the proPO cascade is organized and activated, but our experiments reported here provide a more detailed study of the activation of two different proPOs and their activating factors.

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