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Manduca sexta hemolymph protease-1, activated by an unconventional non-proteolytic mechanism, mediates immune responses

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ABSTRACT

Tissue damage or pathogen invasion triggers the auto-proteolysis of an initiating serine protease (SP), rapidly leading to sequential cleavage activation of other cascade members to set off innate immune responses in insects. Recently, we presented evidence that *Manduca sexta* hemolymph protease-1 zymogen (proHP1) is a member of the SP system in this species, and may activate proHP6. HP6 stimulates melanization and induces antimicrobial peptide synthesis. Here we report that proHP1 adopts an active conformation (*) to carry out its function, without a requirement for proteolytic activation. Affinity chromatography using HP1 antibodies isolated from induced hemolymph the 48 kDa proHP1 and also a 90 kDa band (detected by SDS-PAGE under reducing conditions) containing proHP1 and several serpins, as revealed by mass spectrometric analysis. Identification of tryptic peptides from these 90 kDa complexes included peptides from the amino-terminal regulatory part of proHP1, indicating that proHP1* was not cleaved, and that it had formed a complex with the serpins. As suicide inhibitors, serpins form SDS-stable, acyl-complexes when they are attacked by active proteases, indicating that proHP1* was catalytically active. Detection of *M. sexta* serpin-1, 4, 9, 13 and smaller amounts of serpin-3, 5, 6 in the complexes suggests that it is regulated by multiple serpins in hemolymph. We produced site-directed mutants of proHP1b for cleavage by bovine blood coagulation factor Xa at the designed proteolytic activation site, to generate a form of proHP1b that could be activated by Factor Xa. However, proHP1b cut by Factor Xa failed to activate proHP6 and, via HP6, proHP8 or proPAP1. This negative result is consistent with the suggestion that proHP1* is a physiological mediator of immune responses. Further research is needed to investigate the conformational change that results in conversion of proHP1 to active proHP1*.

1. Introduction

Serine protease (SP) pathways are conserved in evolution as a strategy to mediate rapid defense responses to tissue damage or microbial infection (Jiang and Kanost, 2000; Krem and Di Cera, 2002). In such cascade pathways, a series of preexisting, inactive zymogens become active SPs through specific recognition and sequential proteolysis. The potency and duration of these responses are often controlled by SP inhibitors including serpins (Gubb et al., 2010; Meekins et al., 2017). Serpins are suicide inhibitors that form stable acyl-enzyme complexes with cognate proteases, only when the active SPs attack the serpins at their reactive center loop (Gettins, 2002). In the last two decades, genetic and biochemical studies have generated much knowledge on the constitution and regulation of immune SP pathways in *Drosophila*, mosquitoes, beetles, moths, and other insects (Barillas-Mury, 2007; Jiang et al., 2010; Park et al., 2010; Veillard et al., 2016). Many pathway members contain an amino-terminal regulatory clip domain, a linker, and then an SP catalytic domain (Kanost and Jiang, 2015). After cleavage, the catalytic domain remains attached to the clip domain and linker via an interchain disulfide bridge. Clip-domain serine protease homologs (SPHs) also participate in insect immune

Abbreviations: AMP, antimicrobial peptide; βGRP1/2, β1,3-glucan recognition protein-1/2; DAP, diaminopimelic acid; DHI, 5,6-dihydroxyindole; MBP, microbe binding protein; NP and IP, cell-free hemolymph from naïve or induced larvae; CPC, cetylpyridinium chloride; DTT, dithiothreitol; HP, hemolymph (serine) protease; HP1, formerly hemocyte protease-1; PO and proPO, phenoloxidase and its precursor; PAP, proPO activating protease; PGRP1, peptidoglycan recognition protein-1; SP and SPH, serine protease and its non-catalytic homolog; TCEP, tris(2-carboxyethyl) phosphine.

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responses (*e.g.* phenoloxidase (PO) mediated melanization), but the cleavage occurs between Cys-3 and Cys-4 of the clip domain in these SPHs (Kwon et al., 2000; Wang and Jiang, 2004; Yu et al., 2003).

Melanization pathway has been well studied in *Manduca sexta* (Kanost and Jiang, 2015). A modular serine protease, HP14, is auto-activated in the presence of β-1,3-glucan and its recognition protein βGRP1/2 (Wang and Jiang, 2006, 2010) or in the presence of diaminopimelic acid-peptidoglycan (DAP-PG), microbe-binding protein (MBP), and PG recognition protein-1 (PGRP1) (Wang and Jiang, 2017). Active HP14 then cuts proHP21 to form HP21 (Wang and Jiang, 2007), which in turn activates proPAP2 and proPAP3 (PAP for proPO activating protease) (Gorman et al., 2007). ProPAP1 is activated by HP6 (An et al., 2009). PAP1, PAP2 or PAP3 cleaves proPO to PO in the presence of a complex of SPH1 and SPH2. The proHP6 cleavage activation may involve proHP1 (Yang et al., 2016). Besides proPAP1, HP6 cuts proHP8 to form HP8, HP8 activates pro-Spätzle, and Spätzle binds a Toll receptor to trigger an intracellular pathway that induces the synthesis of antimicrobial peptides (AMPs) (An et al., 2009, 2010).

Proteolytic processing is a common, posttranslational modification and, in the case of zymogen activation, it rapidly converts inactive precursors to active enzymes. Zymogen activation can also occur in the absence of an activating protease. Prophenoloxidases (proPOs), isolated from the tobacco hornworm *Manduca sexta*, are activated by cetylpyridinium chloride (CPC, a cationic detergent) (Hall et al., 1995). Concentration of proPAP1 in a centrifugal device produced some active PAP1 (*i.e.* proPO activating protease-1), even though PAP1 does not usually activate proPAP1 by itself (Wang et al., 2001).

We recently reported the CPC-induced auto-proteolysis of proHP1, which can then cleave proHP6 (Yang et al., 2016). HP1 is the first clip-domain SP cloned from *M. sexta* and a member of the CLIPD subfamily (Cao et al., 2015; Jiang et al., 1999; Yang et al., 2016). ProHP1 is present in plasma at an estimated concentration of 27 μg/ml as a mixture of two closely related proteins, proHP1a and proHP1b (89.7% identical; 95.1% similar), at a ratio of approximately 7:1. For simplicity, we use proHP1* to denote the active proHP1 in plasma after microbial elicitation. The proenzymes are produced in granular hemocytes but not in fat body, and injection of bacteria into larvae led to differential expression of the two genes (Yang et al., 2016). Incubation of CPC with proHP1a/b or catHP1a/b (the catalytic domain) yielded an amidase activity that hydrolyzed *N*-acetyl-Leu-Asp-Leu-His-*p*-nitroanilide (LDLH*p*Na), a substrate synthesized to match the sequence preceding the proteolytic activation site of *M. sexta* proHP6 (An et al., 2009). The recombinant proHP1a/b or catHP1a/b elicited proPO activation in cell-free hemolymph, and catHP1a-treated plasma contained the proteolytically activated forms of HP6, HP8, and PAP1 (Yang et al., 2016). Based on these results, we hypothesize that proHP1* may be a physiological activator of proHP6.

Another line of evidence for the current hypothesis arose from the study of *M. sexta* serpin-4 and -5, which suppress proPO activation in plasma by forming covalent complexes with HP1, HP6, and other HPs (Tong et al., 2005; Tong and Kanost, 2005). The complex of HP1 and serpin-4 migrated as a 90 kDa band in SDS-PAGE under reducing condition, substantially higher than 75 kDa or the typical size of a serpin-protease complex. This observation led us to test whether the 90 kDa band contains a region before the predicted cleavage activation site of proHP1 [(A/P)QGR↓VF(G/D)S]. In addition, we substituted AQGR of proHP1a (or PQGR of proHP1b) with IEGR − the recognition sequence of bovine blood coagulation factor Xa, to test whether proHP1a/b mutant cleaved by Factor Xa can function as a proHP6 activating enzyme. We also tested a hypothesis that α-helical antimicrobial peptides (detergent-like AMPs) or other plasma proteins may mimic the role of CPC by inducing the proHP1* conformation that can cleave proHP6 at the LDLH↓ILGG site. To address these questions, we have performed a series of experiments, demonstrating the existence of proHP1*, and discuss implications of the positive and negative results.

2. Materials and methods

2.1. Insect rearing, immune challenge, and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Each day 2, 5th instar larva was injected with a mixture of killed bacteria (Yang et al., 2016). Induced hemolymph (IH) was collected from cut prolegs of three larvae 24 h later and centrifuged to remove hemocytes. Similarly, control hemolymph (CH) samples were prepared from naïve day 2, 5th instar larvae.

2.2. Immuno-affinity purification of proHP1 and its associated proteins

Cell-free hemolymph samples (10 ml) from immune-challenged larvae were incubated with *Micrococcus luteus* (100 μg) for 30 min at room temperature to activate the SP-SPH system. To avoid melanization and protein crosslinking, 10 mM diethylthiocarbonate (a Cu^{2+} chelator that inhibits PO) and 1 mM 1-phenyl-2-thiourea (another PO inhibitor) were added to the reaction mixture. Rabbit antiserum against HP1 (4.8 ml) was coupled to 2.4 ml Protein A-Sepharose beads (Sigma) according to the manufacturer's instructions. For isolating serpin-HP1 complexes, the plasma activated by *M. luteus* was mixed with the antibody-coupled beads for 8 h at 4 °C with gentle agitation. The suspension was loaded into a Poly-Prep column (Bio-Rad) and then washed with 20 ml of 1 M NaCl and 20 ml of 10 mM sodium phosphate, pH 6.8 to remove unbound proteins. Bound proteins were eluted from the column with 50 mM glycine-HCl, pH 2.5. Fractions (0.5 ml each) were instantly neutralized with 50 μl, 1 M Tris-HCl, pH 8.0 in each collection tube. The elution fractions were separated by SDS-PAGE, followed by light staining with Coomassie Blue or immunoblot analysis.

2.3. In-gel trypsinolysis and LC-MS/MS analysis

The 80−95 kDa region excised from the SDS-PAGE gel described above was cut into small pieces before extensive destaining with 50% acetonitrile in 50 mM NH_4HCO_3, pH 8.0. The proteins in the gel pieces were reduced with tris(2-carboxyethyl) phosphine (TCEP), alkylated by iodoacetamide at Cys residues, and digested with sequencing grade trypsin for 16 h at 37 °C, as described before (He et al., 2016). The tryptic peptides were extracted from the gel pieces using 1% trifluoroacetic acid for LC-MS/MS analysis on an LTQ-OrbitrapXL mass spectrometer (Thermo Scientific) in the DNA/Protein Resource Facility at Oklahoma State University. The MS/MS spectra were searched against Msexta_060614.fasta (He et al., 2016) for protein identification. Centroided ion masses were extracted by extract_msn.exe utility from Bioworks (v3.3.1) for database searching. Scaffold (v4.2.0, Proteome Software Inc.) was used to validate MS/MS-based peptide or protein identifications. Scan numbers were extracted from Scaffold Viewer to locate HPLC peaks of specific tryptic peptides. Xcalibur Qual Browser (v3.0 Thermo Scientific) was used to find the retention time along with base peak from the raw file. To generate a peak map, parameters were set to: mass tolerance (10.0 ppm); scan filter (FTMS + p NSI full ms [360.00−1400.00]; plot type (base peak); detector (MS);