Interaction of β-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in Manduca sexta*

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Running title: M. sexta proHP14 activated by β-1,3-glucan and βGRP2

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A serine proteinase pathway in insect hemolymph leads to prophenoloxidase activation, an innate immune response against pathogen infection. In the tobacco hornworm Manduca sexta, recombinant hemolymph proteinase 14 precursor (proHP14) interacts with peptidoglycan, autoactivates, and initiates the proteinase cascade (1). Here, we report the purification and characterization of proHP14 from the hemolymph of bacteria-injected M. sexta larvae. The zymogen, consisting of a single polypeptide with a molecular mass of 68.5 kDa, is truncated at the amino terminus. It is converted to a two-chain active form in the presence of β-1,3-glucan (a fungal cell wall component) and β-1,3-glucan recognition protein-2. The 45 kDa heavy chain contains four low-density lipoprotein receptor A repeats, one Sushi domain, and one unique cysteine-rich region, whereas the 30 kDa light chain contains a serine proteinase domain, which was labeled by ³H-diisopropyl fluorophosphate. ProHP14 in the plasma strongly binds curdlan, zymosan and yeast, and also interacts with peptidoglycan and Micrococcus luteus. Addition of autoactivated HP14 elevated phenoloxidase activity level in the larval plasma. Recombinant M. sexta serpin-II reduced prophenoloxidase activation by inhibiting HP14. These data are consistent with the current model on initiation and regulation of the prophenoloxidase activation cascade upon recognition of pathogen-associated molecular patterns by specific pattern recognition proteins.

Innate immunity is essential for the wellbeing of vertebrates and invertebrates. Key features of this defense system include: pattern recognition of infectious agents, stimulation of extra- and intracellular signaling pathways, immediate cellular/humoral responses, and induced synthesis of immune factors (e.g., antimicrobial peptides) (2-4). Serine proteinase cascades in plasma, such as the human complement system and horseshoe crab hemolymph coagulation pathway, mediate many of the defense responses (5,6). In insects, extracellular serine proteinases generate spätzle, phenoloxidase (PO)¹, and plasmatocyte spreading peptide via limited proteolysis (5,7). In Drosophila, a proteinase cascade activates spätzle to induce antimicrobial peptide synthesis by the Toll pathway. While Persephone (a clip-domain serine proteinase) is a component of the cascade (8), its activating proteinase and protein substrate remain unclear. Neither is it known how Gram-positive bacteria trigger the cascade activation (9) or how melanization often associates with malfunctioning of this proteinase pathway (10-12).

Microbial infection or tissue damage often leads to proteolytic activation of prophenoloxidase (proPO) in insects. Active PO catalyzes the formation of quinones, reactive intermediates for melanization (13-15). Quinones may also participate in wound
healing and sequestration of parasites or pathogens. To minimize the cytotoxicity of quinones to host tissues/cells, conversion of proPO to PO occurs as a local reaction involving a system of interacting proteins (e.g., pattern recognition receptors, serine proteinases, serine proteinase homologs, serpins, and proPO). While some of these molecules have been characterized at the molecular level, the constituents, order, initiation, and regulation of the proteinase pathway are still poorly understood. Our current knowledge is mainly limited to pathogen recognition and proPO activation—the first and last steps of the cascade (7,16).

Recently, we reported the cDNA cloning, recombinant expression and functional elucidation of M. sexta HP14, an immune-responsive hemolymph proteinase (1). This Sushi-domain enzyme contains seven Cys-rich regulatory domains and a serine proteinase domain. Incubation with peptidoglycan leads to auto-processing of the recombinant proHP14 and generation of an amidase activity. Supplementation of hemolymph with the zymogen enhanced proPO activation in response to M. luteus. These data suggest that proHP14 binds to the Gram-positive bacteria, autoactivates, and triggers the immune proteinase pathway in M. sexta. To further explore the physiological function of HP14, we purified and characterized proHP14 from the hemolymph of M. sexta larvae injected with microbes. In the presence of a fungal cell wall component and its recognition protein, autoproteolysis of proHP14 yielded active HP14 that enhanced proPO activation in the plasma. Its binding to microbial surface components and inhibition by M. sexta serpin-I1 were also investigated.

**Experimental Procedures**

*Insect rearing, bacterial challenge, and hemolymph collection—* M. sexta eggs were purchased from Carolina Biological Supply. The larvae were reared on an artificial diet (17). Day 2, 5th instar larvae were injected with a mixture of formaldehyde-killed *Escherichia coli* (3 × 10^7 cells), *M. luteus* (30 µg) and curdlan (30 µg) in 50 µl H_2O. Hemolymph was collected from cut prolegs of the larvae at 24 h after the immune challenge. Individual hemolymph samples were immediately mixed with equal volume of pH 7.0, 100% saturated (NH_4)_2SO_4 to prevent melanization.

**Proteins, microorganisms, and microbial polysaccharides—** M. sexta β-1,3-glucan recognition protein-2 (βGRP2), immulectin-2, and serpin-I1 were isolated as described previously (18-20). M. luteus (2 × 10^9 cells/ml), *E. coli* (2 × 10^9 cells/ml) and *S. cerevisiae* (5 × 10^8 cells/ml) were treated with 0.4% formaldehyde at room temperature for 2 days and thoroughly washed with sterilized 0.85% NaCl. Curdlan (from *Alcaligenes faecalis*), laminarin (from *Laminaria digitata*), lipopolysaccharide (from *E. coli* O127:B8), lipoteichoic acid (from *Streptococcus faecalis*), peptidoglycan (from *Staphylococcus aureus*), and zymosan A (from *S. cerevisiae*) were purchased from Sigma. These microbial cells and cell wall components were heated at 100°C for 5 min to inactivate possible contaminating proteinases.

**Purification of proHP14 from induced M. sexta hemolymph—** All procedures for proHP14 purification were carried out at 4°C using buffers filtered through a 0.22 µm membrane. The induced hemolymph sample (55 ml, stored at -80°C) was thawed as 50% (NH_4)_2SO_4 suspension. After centrifugation at 17,400×g for 20 min, the protein precipitate was collected and dissolved in 50 ml of buffer A (1 mM benzamidine, 0.01% 1-phenyl-2-thiourea, 0.5 M NaCl, 10 mM potassium phosphate, pH 6.8). The solution was centrifuged at 17,400×g for 30 min to remove flocculent materials. Saturated (NH_4)_2SO_4 solution was slowly added to the supernatant to a final saturation of 35%. After centrifugation, the precipitate was collected and dissolved in 15 ml buffer A.

For rapid removal of (NH_4)_2SO_4, the protein sample was passed through a Sephadex
G25 column (2.5 cm i.d.×16 cm) containing buffer A. Protein fractions were pooled (~40 ml) and applied to a hydroxylapatite column (2.5 cm i.d.×7 cm, Bio-Rad) equilibrated with the same buffer. After washing with 100 ml buffer A, bound proteins were eluted with a linear gradient of 10-150 mM potassium phosphate in buffer A at 0.5 ml/min for 6 h. Fractions were analyzed by SDS-PAGE and immunoblotting using HP14 antibody. The flow-through fractions were combined and precipitated with 50% saturated (NH₄)₂SO₄. Following centrifugation, the pellet was dissolved in 3 ml buffer B (pH 7.5, 20 mM Tris-HCl, 0.5 M NaCl, 0.01% Tween-20, and 1 mM benzamidine) and separated on a Sephacryl S100-HR column (2.5 cm i.d.×120 cm) equilibrated with buffer B.

The proHP14 fractions were combined, supplemented with 1 mM CaCl₂ and MgCl₂, and loaded on a concanavalin A-Sepharose column (5 ml). After washing, proHP14 was eluted from the lectin column with buffer B containing 1 mM CaCl₂, 1 mM MgCl₂, and 0.4 M methyl α-D-mannopyranoside. Eluted proteins were diluted with 10 volumes of buffer C (0.01% Tween-20, 2 mM benzamidine, 10 mM potassium phosphate, pH 6.4) and applied to a dextran sulfate-Sepharose CL-6B column (5 ml) (21). After washing with 30 ml of buffer C, a linear gradient of 0-1.0 M NaCl in buffer C was employed to elute the bound proteins at 1 ml/min for 30 min. Twenty fractions of 1.5 ml were collected and analyzed by SDS-PAGE. The purified proHP14 was stored at -80°C before use. Prior to functional analyses, the purified protein was passed through a Sephadex G25 column (1.5 cm i.d.×15 cm) equilibrated with pH 7.5, 20 mM Tris-HCl, 20 mM NaCl to remove benzamidine.

Characterization of M. sexta proHP14 – MALDI-TOF mass spectrometry of proHP14 was carried out at Nevada Proteomics Center. Briefly, the purified protein (2 µg) was separated by 10% SDS-PAGE under reducing condition and stained with Coomassie blue. Individual protein bands were excised from the gel, treated with 100 mM iodoacetamide, and digested with sequencing-grade trypsin (25 µl, 5 ng/µl) (Promega) in 25 mM ammonium bicarbonate at 37°C for 16 h (22). In a parallel experiment, gel slices containing 2 µg proHP14 were reacted with iodoacetamide and then 100 ng Glu-C (Princeton Separation) at 30°C for 16 h. After desalting with a ZipTip C18 (Millipore), the peptides were analyzed on an ABI 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems). Mass data were acquired in reflector mode within the range of 800 – 4,000 Da, and 2500 laser shots were averaged for each mass spectrum. Molecular masses of the peptides from proHP14 were calculated (http://prospector.ucsf.edu/) and compared with those of observed monoisotopic peaks to locate their positions. The isoelectric point of proHP14 was measured on a precast PhastGel IEF-9 (Amersham Pharmacia Biotech) (23).

Cleavage activation of proHP14 induced by recognition of microorganisms – Purified proHP14 (200 ng, 10 µl), microbial cells or microbial cell wall components (10 µg, 1 µl), corresponding recognition proteins (40 ng, 1 µl), CaCl₂ (100 mM, 1 µl), and buffer D (20 mM Tris-HCl, 20 mM NaCl, 5 mM CaCl₂, pH 8.0, 2 µl) were incubated at 37°C for 1 h. The reaction mixtures (8 µl) were analyzed by 12% SDS-PAGE followed by silver staining or immunoblotting to reveal possible proteolytic cleavage.

³H-diisopropyl fluorophosphate labeling of the active site serine residue in HP14 – ProHP14 (200 ng, 10 µl) was incubated with curdlan (10 µg, 1 µl), βGRP2 (40 ng, 2 µl), ³H-DFP (1 µl, 0.1 mM, 10 mCi/mmol, New England Nuclear), CaCl₂ (100 mM, 1 µl) and buffer D (2 µl) at 37°C for 1 h. The reaction mixture (17 µl), along with ¹⁴C-labeled Mr standards, was resolved by 12% SDS-PAGE under reducing condition. After 2,5-diphenyloxazole treatment (24), the gel was dried and exposed to an X-ray film for 30 days at -80°C prior to development.

Optimization of conditions for proHP14
autoactivation – The general conditions for curdlan-induced proHP14 activation were: purified proHP14 (200 ng, 10 µl), curdlan (10 µg, 1 µl), βGRP2 (40 ng, 2 µl), CaCl2 (100 mM, 1 µl), and buffer D (2 µl) incubated at 37°C for 1 h. One of these conditions was changed as specified in Fig. 3 to test the effects of βGRP2/curdlan amount, incubation time/temperature/pH, and NaCl concentration. To assess cleavage extent, the reaction mixtures (8 µl) were separated by 10% SDS-PAGE under reducing condition and visualized by silver staining. The effect of pH was investigated by replacing buffer D with 3 µl, 1:5 diluted Polybuffer 96 (Amersham Biosciences) adjusted to various pH.

Determination of the proteolytic cleavage site in HP14 – HP14 generated under the optimal conditions was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After staining with Ponceau S (Sigma), the catalytic chain of HP14 was subjected to automated Edman degradation at Harvard Microchemistry Facility.

Binding of plasma proHP14 to microorganisms – Following 0-35% ammonium sulfate fractionation and centrifugation, hemolymph (0.5 ml) from the injected larvae was dissolved in 200 µl, pH 7.5, 20 mM Tris-HCl, 0.5 M NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.01% 1-phenyl-2-thiourea. The plasma fraction was incubated with M. luteus (2×10^9 cells), E. coli (2×10^9 cells), S. cerevisiae (5×10^7 cells), or microbial cell wall components (1 mg, 100 µl) at room temperature for 30 min. After centrifugation at 13,000×g for 1 min, the supernatants were removed. The pellets were washed five times with 50 mM Tris-HCl, 5 mM CaCl2, pH 7.8. Bound proteins were eluted with 40 µl, 2×SDS sample buffer at 100°C for 5 min. As controls, the microbial cells/polysaccharides only were treated similarly. The supernatants, bound proteins, and control samples were subjected to 10% SDS-PAGE and immunoblot analysis using 1:2000 diluted HP14 antiserum as the first antibody. Distribution of proHP14 in the supernatant and pellet was estimated by comparing relative intensities of the immunoreactive bands.

Binding of purified proHP14 to microorganisms – Purified proHP14 (1 µg, 50 µl) and CaCl2 (100 mM, 5 µl) were incubated with microbial cells or cell surface components in the presence or absence of their binding proteins (200 ng, 10 µl) at 37°C for 60 min. The total volume was adjusted to 65 µl with buffer D. As described above, the supernatants and bound proteins were obtained and subjected to SDS-PAGE followed by silver staining or immunoblot analysis. Microbial cells or cell wall components, proHP14, and recognition proteins were included as controls.

Stimulation of proPO activation system by HP14 – ProHP14 (1 µg, 50 µl), curdlan (1 mg pellet), βGRP2 (200 ng, 10 µl), and CaCl2 (100 mM, 5 µl) were incubated at 37°C for 90 min. After centrifugation, 2 µl of the supernatant was incubated with plasma (1:10 diluted in buffer D, 5 µl) and buffer D (10 µl) on ice for 20 min. PO activity in the reaction mixture was assayed using dopamine as a substrate (25). The pre-incubation mixture without proHP14 or curdlan-βGRP2 was used as negative controls.

Inhibition of HP14-induced PO activation by serpin-II – To detect the formation of an SDS-stable complex of HP14 and serpin-II, purified proHP14 (200 ng, 10 µl), serpin-II (3 µg, 1 µl), curdlan (10 µg, 1 µl), βGRP2 (40 ng, 2 µl), CaCl2 (100 mM, 1 µl) were incubated at 37°C for 90 min. In the controls, proHP14 or serpin-II was replaced by the same volumes of buffer D. The reaction mixture and negative controls (10 µl) were separated by 7.5% SDS-PAGE and subjected to immunoblot analysis using 1:1000 diluted serpin-I antiserum. To examine the effect of serpin-II on proPO activation induced by HP14, the active proteinase was generated under the optimal conditions. After centrifugation, supernatant (2 µl), serpin-II (3 µg, 1 µl) and buffer D (9 µl) were incubated on ice for 10 min. Induced plasma (5 µl, 1:10 diluted) was added to the HP14-serpin-II mixture and further incubated...
on ice for 20 min prior to PO activity assay. A mixture of the supernatant (2 µl), buffer D (10 µl) and induced plasma (5 µl, 1:10 diluted) was included as a positive control, whereas plasma only was a negative control.

Results

Purification and characterization of M. sexta proHP14 from the induced larval hemolymph – Using polyclonal antiserum against HP14, we detected a protein doublet in the hemolymph, whose level increased after an immune challenge (1). The immunoreactive proteins with apparent molecular masses of 75 and 67 kDa were mostly found in 0-35% ammonium sulfate fraction of the induced plasma. After desalting and buffer exchange, we resolved the fractionated hemolymph by hydroxyl-apatite chromatography. The flow-through contained the large protein, whereas the bound part included the small one (Fig. 1, A and B). Because the secreted recombinant proHP14 had an apparent $M_r$ of ~75,000 (1), we decided to isolate proHP14 from the unbound fractions, representing ~20% of the total proteins loaded onto the hydroxyl-apatite column. Following a concentration step, the pooled flow-through fractions were separated by gel filtration chromatography on a Sephacryl S100-HR column. About 85% of the proteins were removed in this step. The combined proHP14 fractions were loaded onto a concanavalin A-Sepharose column. After nearly half of the loaded proteins were removed, proHP14 and other glycoproteins were eluted from the lectin column. The pooled proHP14 fractions were resolved by ion exchange chromatography on a dextran sulfate column. ProHP14 eluted from the column at ~0.25 M NaCl. With ~7×10^3-fold purification, 179 µg of proHP14 was isolated from 55 ml of induced hemolymph. As judged by SDS-PAGE analysis, the proenzyme was essentially pure and it migrated to the 75 kDa position under reducing condition (Fig. 1B).

We determined by MALDI-TOF mass spectrometry that the molecular mass of “75 kDa” proHP14 from the induced plasma was actually 68,545 ± 69 Da, smaller than the theoretical value of the mature proenzyme (residues 1-649, 71,774 Da)(1). Peptide mass fingerprint analysis indicated that 28 predicted trypsinolytic fragments (800 – 4000 Da) were apparent on the mass spectrum, covering 59% of the sequence from residue 68 to the carboxyl terminus (Table 1). The identification of F^644^W TDEY^649^ demonstrated that the proenzyme was intact at the carboxyl terminus and, therefore, truncated at the amino terminus to account for the mass reduction. Due to amino terminal blocking, automated Edman degradation failed to yield any sequence from proHP14. However, we detected two peptides (760.34 and 949.46 Da) that are identical in mass to pyroGlu^62^LSNCR^67^ (760.33 Da, carboxyamidomethyl Cys (CAM-C)) and I^68^SQWQC (949.457 Da, CAM-C). Right before Gln^62^, there is a recognition site of intracellular processing enzymes: Arg^58^-Ser-Arg-Arg^61^ (26).

Was proHP14 truncated somewhere before Gln^62^ and, after trypsin cleavage at Arg^61^, the exposed Gln^62^ spontaneously became pyroGlu^62^? Although it is possible, evidence suggests this is not the case: 1) there is no other known processing site in the 1st LDLrA repeat or in the following linker (residues 44-65); 2) the 1st Gln in Q^106^CQYNWFR^113^ did not become pyroGlu^106^ under the same condition (Table 1); 3) Glu-C digestion generated a peptide (1805.835 Da) with a mass identical to that of pyroGlu^62^LSNCRISQWQCKD (1805.802 Da, 2 CAM-C). Therefore, the “75 kDa” proHP14 starts at pyroGlu^62^ and Gln^62^ -> pyroGlu^62^ conversion occurred before trypsin digestion.

The calculated mass of proHP14 (residues 62-649) is 65,183 Da, smaller than the experimental value (68,545 Da). Since proHP14 binds concanavalin A, glycosylation appears to be responsible for the mass difference of 3,362 Da. We predict that the posttranslational modification occurs at N^145^ET, N^383^GT, and/or N^582^GT, because peptide...
masses containing these were not found (Table 1). The isoelectric point of proHP14 was determined to be 5.7, slightly higher than the calculated value (5.5) from its amino acid sequence (residues 62-649).

Cleavage activation of proHP14 induced by microorganisms and their binding proteins

To test whether or not microorganisms could activate proHP14, we incubated purified proHP14 with various microbial cells or cell surface molecules in the presence/absence of their binding proteins. SDS-PAGE analysis indicated that proHP14 was cleaved after incubating with yeast, curdlan, or zymosan A in the presence of βGRP2 (Table 2). However, laminarin (a soluble form of β-1,3-glucan) did not lead to proHP14 cleavage activation. E. coli did not cause such a change with or without immulectin-2, and nor did M. luteus.

While incubation of proHP14 with peptidoglycan did lead to proHP14 cleavage, the efficiency was lower than with β-1,3-glucan and βGRP2.

Proteolytic processing of proHP14 occurred when insoluble β-1,3-glucan and βGRP2 were present at the same time (Fig. 2A). Since proHP14 cleavage did not happen after incubation with curdlan or βGRP-2 alone, binding-induced autoactivation (rather than a contaminating proteinase) is responsible for the limited proteolysis. Cleaved HP14 was separated into 45 and 30 kDa bands by SDS-PAGE under reducing condition whereas, under nonreducing condition, HP14 migrated as a single band at around 70 kDa (Fig. 2B). This agrees with our prediction based on the deduced sequence and domain structure of HP14: an interchain disulfide bond links the 30 and 45 kDa polypeptides (1). While the 45 kDa heavy chain was blocked at the amino terminus, we determined the first ten amino acid residues of the light chain as: Val-Leu-Gly-Gly-Arg-Ala-Gln-Phe-Gly. This perfectly matched the sequence after the predicted proteolytic activation site (Gly-Thr-Glu-Leu*Val-Leu-Gly-Gly). Affinity labeling by 3H-DFP demonstrated that the light chain was catalytically active (Fig. 2C). In other words, the 30 kDa band represents the proteinase domain at the carboxyl terminus.

We optimized the reaction conditions for proHP14 activation. At 15 min after 200 ng proHP14 was incubated with 10 µg curdlan and 10 ng βGRP2, nearly half of the proenzyme was converted to the two-chain active HP14 (Fig. 3). The cleavage reaction was >90% complete at 45 min. Further increase in βGRP2 or curdlan level had little effect on the proteolytic activation. Ionic strength of the reaction mixture negatively impacted the molecular interactions: 80% of the cleavage was blocked in the presence of 200 mM NaCl. KCl, Na2SO4, and NaAc had a similar effect (data not shown). The optimum pH for proHP14 autoactivation was around 7.5-8.5, and the cleavage was more complete at 37°C than 25°C (data not shown). At the physiological pH (6.7) and ionic strength (equivalent of 200 mM NaCl) (27), the proteolysis in the larval plasma is estimated to occur at 15% of the optimal rate determined in vitro.

Binding of proHP14 to microorganisms in the presence or absence of plasma proteins

To better understand the role of proHP14 in immune responses, we tested its binding with various microbial cells or cell surface molecules using 0-35% ammonium sulfate fraction of the plasma. In the presence of high salt and 1-phenyl-2-thiourea, proHP14 activation and protein crosslinking was prevented. ProHP14 in the fractionated hemolymph bound to the Gram-positive bacteria and yeast but not E. coli (Fig. 4B). Peptidoglycan and β-1,3-glucan appeared to be responsible for the binding. While the reduction of proHP14 in M. luteus-, and peptidoglycan-treated supernatants were relatively small, strong interactions between proHP14 and curdlan/zymosan completely depleted the proenzyme in the solution phase (Fig. 4A). The binding of proHP14 to S. cerevisiae greatly reduced the proenzyme in the supernatant.
We further examined whether the purified proHP14 can directly associate with microbes in the presence or absence of their recognition proteins. Immunoblot analysis showed that most proHP14 remained in supernatants treated with *E. coli*, *M. luteus*, or *S. cerevisae* (Fig. 5A and Table 3). Since supplementing the mixtures with immulectin-2 or βGRP2 did not increase the amount of binding, other plasma proteins must have contributed to the binding of proHP14 to *M. luteus* or yeast (Fig. 4). In the presence of βGRP2, zymosan or curdlan caused larger reduction of proHP14 in the supernatants than yeast did (Fig. 5A). This reduction was partly due to the proteolytic processing of proHP14. While βGRP2 did not seem to enhance the binding of proHP14 to *S. cerevisae*, its coexistence with curdlan induced the proteolytic processing of proHP14 (Fig. 5B).

Initiation of proPO activation system in the larval hemolymph by autocleaved HP14 – To test whether or not HP14 triggers the proPO activation pathway in the plasma, we first used curdlan and βGRP2 to completely activate proHP14 under the optimal conditions (Fig. 3). After removing the elicitor and associated βGRP2 by centrifugation, the supernatant containing the two-chain HP14 was added to plasma from the naïve or bacteria-injected larvae. After incubation, high levels of PO activity developed in the control and induced hemolymph samples (Fig. 6). The enzyme activities (0.06 and 1.21 U) were low in the plasma controls and became slightly higher when proHP14 was added (0.32 and 1.59 U), thus, the proteinase zymogen had only a small effect on proPO activation. In another control, after the supernatant of curdlan and βGRP2 mixture was incubated with the two plasma samples, higher PO activities (0.52 and 3.18 U) were detected, suggesting that the supernatant may contain trace amounts of curdlan and βGRP2 to activate the proteinase cascade. Nevertheless, such activity increases were much lower than those in HP14-treated plasma samples (20.5 and 43.4 U). These results are consistent with the conclusion that HP14 initiated the proPO activation system in the hemolymph.

Inhibition of HP14 by serpin-II – Although 3H-DNP labeling, proHP14 auto-processing and proPO activation demonstrated HP14 is an active serine proteinase, we failed to detect its amidase activity using Ala-Ala-Pro-Leu-p-nitroanilide, as we did with peptidoglycan-activated recombinant HP14 (1). In order to further demonstrate that HP14 is an active proteinase, we decided to test if HP14 could form an SDS-stable complex with serpin-II. Among the 12 variants of *M. sexta* serpin-1, serpin-II has a reactive site sequence (AL*SL) most similar to the proteolytic activation site for HP14 (EL*VL). After curdlan-activated HP14 was incubated with recombinant serpin-II, we detected a complex using serpin-1 antibodies (Fig. 7A). The immunoreactive band, absent in the controls of serpin-1I and HP14 only, had an apparent Mr (~75 kDa) close to that predicted for the complex formed between the HP14 catalytic domain and serpin-II. No such complex was formed with serpin-1J, a negative control with an Arg at the P1 position (data not shown). We further examined the effect of serpin-1I on proPO activation induced by active HP14. As shown in Fig. 6, supplementation of active HP14 greatly enhanced proPO activation in the induced hemolymph. Preincubation of activated HP14 with serpin-1I significantly suppressed proPO activation (Fig. 7B).

Discussion

Innate immunity in vertebrates and invertebrates is activated by host proteins recognizing conserved surface determinants of pathogens (28). Upon binding, these proteins stimulate plasma factors and blood cells to immobilize and kill the invading microorganisms. While many defense reactions are mediated by extracellular serine proteinase pathways (5,6), limited information is available on how pathogen recognition leads to
proteinase activation. A current model suggests that the binding of recognition proteins to a microbial surface induces a conformational change required for interacting with the first proteinase zymogen, leading to its autoactivation and pathway initiation (5,13,29). The lectin-mediated pathway for complement activation in mammals is an example for this model: mannose-binding protein and mannose-binding protein-associated serine proteinase (MASP) trigger the serine proteinase cascade (30). In this paper, we provide biochemical evidence that the pathway for proPO activation in a lepidopteran insect is initiated in similar manner: β-1,3-glucan and βGRP2 together induced proHP14 autoactivation (Fig. 2) and active HP14 by itself started the proPO activation cascade (Fig. 6). These similarities in the proPO activation pathway in arthropods and the complement system in vertebrates suggest that proteinase autoactivation to trigger innate immune responses is an ancient evolutionary adaptation for defense against infection (5,15).

A few known arthropod initiation serine proteinases greatly differ in their domain and subunit organizations. The β-1,3-glucan-responsive proteinases, M. sexta HP14 and T. tridentatus factor G, trigger proPO activation and hemolymph coagulation cascades, respectively (5,6). In the insect, recognition of β-1,3-glucan is performed by a separate protein (βGRP2), whereas in the horseshoe crab, a subunit of factor G executes the same function. This subunit, which associates with the proteinase subunit, is encoded by a separate gene. In contrast, the horseshoe crab factor C consists of a single polypeptide. This lipopolysaccharide-responsive enzyme, by strictest definition, is a pattern-recognition proteinase. While T. tridentatus factor C and M. sexta HP14 both contain a Sushi domain and a catalytic domain, the types of other structural modules (e.g., LDLr A repeat, C-type lectin) are strikingly different and, unlike Factor C, proHP14 does not autoactivate in the presence of lipopolysaccharide.

The HP14 zymogen produced by baculovirus-infected insect cells had two forms: the 87 kDa proHP14 was primarily intracellular, whereas the 75 kDa one was mostly in the medium (1). With an apparent $M_c$, similar to the purified hemolymph proHP14, the latter may also start with pyroGlu$^62$. Indeed, the 75 kDa recombinant protein was blocked at its amino terminus and intact at the carboxyl terminus. In light of these findings, we further speculate that the 87 kDa protein represents the full-length proHP14 with 5 LDLr A repeats. Since the 87 kDa protein was not detected in M. sexta larval plasma, the function of its 1st LDLr A repeat seems dispensable. Orthologs of HP14 in D. melanogaster, A. gambiae, and Apis mellifera contain 4 instead of 5 LDLr A repeats at the amino terminus. On the other hand, the 67 kDa protein in plasma may represent proHP14 with the first two LDLr A repeats removed – antibodies specific for LDLr, Sushi, 7C, or proteinase domain all recognized this band (Wang and Jiang, unpublished data). Structural and functional comparisons of this form of proHP14 with the 75 kDa proHP14 are under active investigation.

The structure-function relations of human MASPs have shed light on our analysis of M. sexta HP14. Corresponding to the amino terminal LDLr A repeats, the CUB-EGF-CUB region in MASP-1 and MASP-2 is responsible for interacting with mannose-binding protein and for forming homodimer (31). The following two Sushi domains in MASP-2 stabilize the structure of the serine proteinase domain at the carboxyl terminus and are directly involved in the enzyme autoactivation (32). In HP14, the 2nd Sushi domain does not exist: between the Sushi and proteinase domains, there is another cysteine-rich region (1). Perhaps, the last (or 7th) Cys in this region forms the interchain disulfide bond with Cys$^{510}$ in the catalytic domain. If proven true, the part that contains the first six cysteine residues may represent the prototype of a unique domain structure stabilized by three disulfide bonds. We tentatively call that “Wonton domain”.
Structural and functional analyses are required to test if the Sushi and Wonton domains take part in the autoactivation of HP14 and interaction with its substrate.

One important feature of the proHP14 autoproteolysis induced by curdlan and βGRP2 is the high specificity: a single cleavage at the predicted activation site yielded two polypeptides (Fig. 3). In contrast, after recombinant proHP14 had been incubated with peptidoglycan, a series of low M_r bands appeared in a time-dependent manner (1). This apparent low specificity could be caused by the lack of particular plasma factors in the activation reaction. In fact, formation of a macromolecular complex containing HP14 and other immune proteins was observed in M. sexta larval plasma incubated with microbial surface components (33,34). Consequently, further studies are needed to test if other proteins (e.g., peptidoglycan recognition proteins) are required for specific cleavage activation of proHP14 by peptidoglycan. Such experiments would further establish the physiological role of HP14 as a common entry point for responses against Gram-positive bacteria and fungi.

We demonstrated that HP14 is an active proteinase that can be trapped in a covalent complex with a suicidal inhibitor (Fig. 7). While this is not to say that HP14 is the physiological target of serpin-II (since, even at a high concentration, serpin-II failed to completely block proPO activation), it did suggest a possible regulatory mechanism for pathway initiation. Moreover, the Leu residue preceding the cleavage site in serpin-II (and HP14) provided important clues for elucidating the protein substrate of HP14. Since HP14 is a chymotrypsin-like enzyme preferring to cleave after Leu, we could test the ability of HP14 to activate several M. sexta hemolymph proteinase precursors (proHP2, proHP16, proHP19, proHP21, and proHP22) that have a Leu at the putative activation site (35). This will be our next step towards elucidating the proPO activation system in M. sexta.

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**Footnotes**

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1 The abbreviations used are: HP14 and proHP14, hemolymph proteinase 14 and its precursor. PO and proPO, phenoloxidase and its precursor; β-1,3-glucan recognition protein-2, βGRP2; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; DFP, diisopropyl fluorophosphate; CAM-C, carboxyamidomethyl cysteine; LDLr, low-density lipoprotein receptor.
### Table 1  Trypsinolytic peptides from 75 kDa proHP14 matched with the deduced sequence

| calculated mass | observed mass | Δ | start | end | missed cleavage | sequence |
|-----------------|---------------|---|-------|-----|-----------------|----------|
| 949.4566        | 949.4631      | -0.0065 | 68 | 74 | 0 | (R) ISQWQCK (D) |
| 1112.4682       | 1112.4788     | -0.0106 | 75 | 84 | 0 | (K) DGSCINFDGK (C) |
| 3284.3353       | 3284.2832     | 0.0521 | 75 | 103 | 1 | (K) DGSCINFDGKCDGIVDCPASDETHALCR (E) |
| 2190.8854       | 2190.8821     | 0.0033 | 85 | 103 | 0 | (K) CDGIVDCPASDETHALCR (E) |
| 1486.6650       | 1486.6813     | -0.0163 | 104 | 113 | 1 | (R) ERQCQYNWFR (C) |
| 1201.5213       | 1201.5363     | -0.0150 | 106 | 113 | 0 | (R) QCQYNWFR (C) |
| 2987.2280       | 2987.2517     | -0.0237 | 115 | 143 | 1 | (R) CTYGACVDTGPNCNGVQDCADNSDELLPR (R) |
| 1877.7394       | 1877.7474     | -0.0080 | 167 | 183 | 0 | (K) HCDGVDACDGSDETLR (S) |
| 2973.2640       | 2973.2065     | 0.0575 | 189 | 213 | 1 | (K) TCLSYLFQCAVGCVDKDSDCNGIR (E) |
| 3148.5093       | 3148.4790     | 0.0303 | 230 | 258 | 1 | (R) NTSQVVPKEACVLPEYEPHEGGYVSVGM^N (K) |
| 2279.0477       | 2279.0491     | -0.0014 | 238 | 258 | 0 | (K) EGACVLPYEPEGGYVSVGMK (N) |
| 2295.0426       | 2295.0591     | -0.0165 | 238 | 258 | 0 | (K) EGAACVLPYEPEHGGYVSVGM^K (N) |
| 2049.9493       | 2049.9507     | -0.0014 | 285 | 301 | 0 | (R) NDVCTCNWGNFTPLPK (C) |
| 990.4896        | 990.4926      | -0.0030 | 310 | 317 | 0 | (K) EVSVEYK (C) |
| 1197.5686       | 1197.5807     | -0.0121 | 318 | 328 | 0 | (K) CAVLSSQGFR (T) |
| 1405.6752       | 1405.6886     | -0.0134 | 346 | 356 | 0 | (R) KPNYYSETTFR (Y) |
| 1789.8406       | 1789.8472     | -0.0066 | 357 | 372 | 0 | (R) YMSCIGAVGA万余元 (C) |
| 1708.8699       | 1708.8761     | -0.0062 | 394 | 408 | 0 | (R) AQFGLPWQAGYITK (N) |
| 979.5100        | 979.5131      | -0.0031 | 444 | 452 | 0 | (K) EYQAYALGM (L) |
| 1087.6053       | 1087.6149     | -0.0096 | 453 | 460 | 0 | (K) LYPWQPQK (M) |
| 1802.9553       | 1802.9653     | -0.0100 | 469 | 483 | 1 | (K) SEIRDHYSPYFLGR (T) |
| 1317.6955       | 1317.7108     | -0.0153 | 473 | 483 | 0 | (K) DIHYSPYFLGR (T) |
| 1093.5893       | 1093.5852     | 0.0041 | 520 | 529 | 0 | (K) EQLYVGLSLGK (V) |
| 1868.9870       | 1868.9882     | -0.0012 | 530 | 547 | 1 | (K) VAGWGIKDEAGNSQVLK (V) |
| 1157.5802       | 1157.5829     | -0.0027 | 537 | 547 | 0 | (K) DEAGNSQVLK (V) |
| 2895.4715       | 2895.4326     | 0.0389 | 551 | 575 | 0 | (K) LPYVDLQCGISQPSQAFRPYITGD (I) |
| 1333.6501       | 1333.6622     | -0.0121 | 589 | 602 | 0 | (K) GDGGGLSFPAPVRN (L) |
| 860.3467        | 860.3444      | 0.0023 | 644 | 649 | 0 | (R) FWTDEY (-) |

Δ: monoisotopic mass in Da. The starting and ending numbers for individual tryptic peptides were based on the deduced amino acid sequence of mature proHP14 (I).

### Table 2  Effect of microbial cells or cell wall components on autoactivation of proHP14

| microbial cells or surface molecules | binding protein | proteolysis of proHP14 |
|-------------------------------------|-----------------|------------------------|
| E. coli                            | immulectin-2    | -                      |
| lipopolysaccharide                  | immulectin-2    | -                      |
| peptidoglycan                       | -               | -                      |
| M. luteus                           | βGRP2           | -                      |
| lipoteichoic acid                   | βGRP2           | -                      |
| S. cerevisiae                       | βGRP2           | +                      |
| laminarin                           | βGRP2           | -                      |
| curdlan                             | βGRP2           | +                      |
| zymosan                             | βGRP2           | +                      |
Table 3  Binding of proHP14 to microbes or their surface polysaccharides

|                  | curdlan | S. cerevisiae | zymosan | M. luteus | E. coli | peptidoglycan |
|------------------|---------|---------------|---------|-----------|---------|---------------|
| proHP14 in plasma (%)<sup>a</sup> | 0       | 25            | 0       | 66        | 100     | 33            |
| purified proHP14 (%)<sup>b</sup>  | 82      | 97            | 97      | 94        | 95      | not determined |

<sup>a</sup>: binding of proHP14 in plasma is represented as % of the total proenzyme in the supernatant. The relative levels of proHP14 were determined by immunoblotting and densitometry of the protein band.

<sup>b</sup>: binding of proHP14 to microbial cells or cell wall components is represented as % of the total purified zymogen in the unbound fraction.

Figure Legends

Fig. 1. SDS-PAGE analysis of proHP14 isolated from the hemolymph of M. sexta larvae injected with bacteria.  (A) Electrophoretic separation and immunoblot detection of proHP14 in 12 µl of flow-through fraction 18 (left) and bound fraction 54 (right) from the hydroxylapatite column.  (B) SDS-PAGE and silver staining of the 75 kDa proHP14 eluted from the dextran sulfate column. The fractions 9-13 (12 µl each, lanes 1-5) were treated with SDS sample buffer containing dithiothreitol, separated on a 12% SDS-PAGE gel, and stained with silver.

Fig. 2. Autoactivation of proHP14 upon exposure to β-1,3-glucan and βGRP2.  (A) The purified proHP14 (200 ng) was incubated with 10 µg curdlan and 40 ng βGRP2 at 37°C for 2 h. After being treated with SDS sample buffer containing dithiothreitol, the reaction mixture and controls were subjected to 12% SDS-PAGE and silver staining.  (B) A duplicate reaction mixture separated by SDS-PAGE under nonreducing condition.  (C) To label the active site Ser residue, the reaction mixture was incubated at 37°C in the presence of <sup>3</sup>H-DFP (1 µl, 0.1 mM, 10 mCi/ mmol). The labeling mixture was resolved by 12% SDS-PAGE under reducing condition and subjected to fluorography (Skinner and Griswold, 1983). a, 75 kDa proHP14; b, βGRP2; c, 45 kDa HP14 heavy chain; d, 30 kDa HP14 light chain.

Fig. 3. Optimization of the reaction conditions for proHP14 auto-processing. The general conditions for proHP14 autoactivation were: proHP14 (10 µl, 20 ng/µl), curdlan (1 µl, 10 µg/µl), and βGRP2 (2 µl, 20 ng/µl) incubated with 20 mM Tris-HCl, pH 7.8, 20 mM NaCl, 5 mM CaCl<sub>2</sub> at 37°C for 90 min. Incubation time (A), βGRP2 amount (B), NaCl concentration (C), or curdlan amount (D) was changed in each experiment. The reaction mixtures were separated by 12% SDS-PAGE under reducing condition and visualized by silver staining. a-d: see Fig. 2 legend.

Fig. 4. Association of microbes and their cell wall components with proHP14 in the fractionated plasma.  Aliquots of 0-35% ammonium sulfate fraction of the induced hemolymph were incubated with M. luteus (lane 1), peptidoglycan (lane 2), E. coli (lane 3), zymosan (lane 4), yeast (lane 5), or curdlan (lane 6) at room temperature for 30 min. After centrifugation at 13,000×g for 1 min, the supernatants (A) were removed for SDS-PAGE under reducing condition. The pellets were washed five times with 50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, pH 7.8. Bound proteins (B) were eluted from the pellet by SDS sample buffer at 100°C for 5 min and resolved by SDS-PAGE. Electrotransfer and immunoblot analysis were performed to detect proHP14 using its antibodies. *: some of the signal came from the cross-reactivity with a co-migrating molecule from M. luteus.
**Fig. 5.** Binding of purified proHP14 to microbes and their surface molecules. Purified proHP14 (1 µg) was incubated with 1 mg heat-treated microbial cells in the presence or absence of their binding proteins (200 ng) in 70 µl, 20 mM Tris-HCl, 20 mM NaCl, 5 mM CaCl₂, pH 7.8, at 37°C for 60 min. After centrifugation at 13,000×g for 1 min, the supernatants (S) were recovered for SDS-PAGE analysis. The pellets were washed five times with 20 mM Tris-HCl, 5 mM CaCl₂, pH 7.8, and the bound proteins (P) were eluted by treating the pellets with SDS sample buffer at 100°C for 5 min. (A) Detection of proHP14 or HP14 binding by immunoblot analysis. For background reduction, 1:1000 diluted HP14 antiserum was preincubated with lysates of *E. coli, M. luteus*, and *S. cerevisae*. *: unknown; Note that the HP14 polyclonal antibodies hardly recognize the 30 kDa catalytic domain (1). (B) Association of proHP14 with curdlan examined by SDS-PAGE and silver staining. a-d: see Fig. 2 legend.

**Fig. 6.** Activation of proPO by autocleaved HP14. Purified proHP14 (1 µg), 1 mg curdlan, and 200 ng βGRP2 were incubated at 37°C for 90 min in 20 mM Tris-HCl, 20 mM NaCl, 5 mM CaCl₂, pH 8.0. After centrifugation, 2 µl of the supernatant was incubated on ice with 17.5 µl buffer and 0.5 µl plasma samples from naïve (A) or bacteria-injected (B) larvae. Plasma alone, plasma and proHP14, as well as a mixture of plasma, curdlan and βGRP2 were included as controls. After 20 min, PO activity was determined using dopamine as a substrate (25) and plotted as mean ± S.E.M. (n = 3) in the bar graph.

**Fig. 7.** Down-regulation of proPO activation by serpin-1I, an HP14 inhibitor. (A) To detect the covalent complex of HP14 and serpin-1I, 200 ng purified proHP14, 10 µg curdlan, and 40 ng βGRP2 were incubated with 3 µg serpin-1I in pH 8.0, 20 mM Tris-HCl, 5 mM CaCl₂ at 37°C for 90 min. The reaction mixture (lane 3) was analyzed by immunoblot analysis using 1:1000 diluted serpin-1 antiserum as the first antibody. The activation mixture (lane 1) and inhibitor alone (lane 2) were run as negative controls. (B) To examine the effect of serpin-1I on proPO activation triggered by HP14, 1 µg proHP14 was first activated by 1 mg curdlan and 200 ng βGRP2 in the presence of 5 mM CaCl₂ at 37°C for 90 min. After centrifugation, the supernatant (2 µl) and serpin-1I (3 µg, 1 µl) were reacted on ice for 10 min in 20 mM Tris-HCl, 5 mM CaCl₂, pH 8.0. Plasma (0.5 µl) from naïve larvae was then added to the HP14-serpin-1I mixture or HP14 (positive control). Plasma alone and a mixture of plasma and serpin-1I were used as negative controls. After 20 min on ice, PO activities in the reaction and controls were measured and plotted as mean ± S.E.M. (n = 3).
Fig. 1

A

B

FT  B

kDa

M  1  2  3  4  5

kDa
Fig. 2
Fig. 3

A  incubation time (min)  0  15  30  45  60

B  βGRP-2 (ng)  20  40  60

C  NaCl (mM)  50  100  200  300  400

D  curdlan (ng)  0  1  10  40
Fig. 5
Fig. 6
Interaction of β-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in *Manduca sexta*

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