Prophenoloxidase-activating Proteinase-2 from Hemolymph of Manduca sexta

A BACTERIA-INDUCIBLE SERINE PROTEINASE CONTAINING TWO CLIP DOMAINS*

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Proteolytic activation of prophenoloxidase in insects is a component of the host defense system against invading pathogens and parasites. We have purified from hemolymph of the tobacco hornworm, Manduca sexta, a new serine proteinase that cleaves prophenoloxidase. This enzyme, designated prophenoloxidase-activating proteinase-2 (PAP-2), differs from another PAP, previously isolated from integuments of the same insect (PAP-1). PAP-2 contains two clip domains at its amino terminus and a catalytic domain at its carboxyl terminus, whereas PAP-1 has only one clip domain. Purified PAP-2 cleaved prophenoloxidase at Arg51 but yielded a product that has little phenoloxidase activity. However, in the presence of two serine proteinase homologs, active phenoloxidase was generated at a much higher level, and it formed covalently linked, high molecular weight oligomers. The serine proteinase homologs associate with a bacteria-binding lectin in M. sexta hemolymph, indicating that they may be important for ensuring that the activation of prophenoloxidase occurs only in the vicinity of invading microorganisms. PAP-2 mRNA was not detected in naïve larval fat body or hemocytes, but it became abundant in these tissues after the insects were injected with bacteria.

Phenoloxidase (PO)1 is implicated in several defense mechanisms in insects, including cuticle sclerotization and melanotic encapsulation (1–3). Quinones produced by phenoloxidase may also participate in wound healing and killing of seque-

tered parasites and pathogens (4). To minimize detrimental effects of the reactive intermediates to host tissues and cells, arthropod phenoloxidases known so far are all produced as inactive proenzymes and require specific proteinases for proteolytic activation.

Activation of proPO in insects is probably mediated by a serine proteinase cascade, analogous to the coagulation pathway and complement system in human plasma (1, 2, 5, 6). Components of this proteinase system in insects may be already present in circulating hemolymph or released from hemocytes or fat body when pathogens or parasites are encountered. Recognition of invading microorganisms or aberrant host tissues may trigger the autoactivation of the first proteinase in the pathway, leading to the sequential activation of other components in the system through limited proteolysis. Prophenoloxidase-activating proteinase (PAP) (also known as prophenoloxidase-activating enzyme (PPAE)) is the terminal enzyme that directly converts proPO to PO. The reactions catalyzed by phenoloxidase can then result in melanization of foreign organisms trapped in capsules or hemocyte nodules (7). Presumably, protein-protein interactions ensure that the defense response occurs near the site of infection. Inhibitors of serine proteinases and phenoloxidases may further reduce unwanted damage caused by the active enzymes (8).

The molecular mechanisms of proPO activation have been investigated in several insect species. We have isolated a PAP from a cuticular extract of the tobacco hornworm, Manduca sexta (9). This serine proteinase, now renamed PAP-1, hydrolyzes a synthetic peptidyl-p-nitroanilide substrate but requires another protein factor for generating active PO. A CDNA clone for PAP-1 was obtained from an M. sexta hemocyte library. Sequence comparison indicated that the protein belongs to a family of arthropod serine proteinases containing a clip domain (10, 11).

In this paper, we report the purification and characterization of a second serine proteinase from M. sexta hemolymph that activates proPO. To distinguish it from PAP-1 isolated previously from cuticles (9), we designate this new proteinase PAP-2. Molecular cloning of PAP-2 indicates that it is most similar to the silkworm PPAE and has two clip domains at its amino terminus (12). The PAP-2 zymogen is present at a higher level in hemolymph of M. sexta larvae that were challenged with killed bacteria. PAP-2 requires serine proteinase homologs from M. sexta plasma (13) as cofactors for proPO activation. Since the serine proteinase homologs associate with immulectin-2, a C-type lectin isolated from Manduca hemo-

lymph (13–15), they may serve as anchoring/auxiliary factors for PAP-2 so that proPO activation only occurs as a local defense response against nonself.
EXPERIMENTAL PROCEDURES

Insects and Hemolymph Collection—M. sexta eggs were originally purchased from Carolina Biological Supply, and larvae were reared on an artificial diet (18). Pharate pupae and cuticular teneral form mitochondria were chilled and dissected for hemolymph collection. When all tissues were cautiously removed from the integument with a spatula, hemolymph was pooled and collected carefully with a 1-mL pipette, avoiding contaminating tissue fragments. This method yields significantly greater amounts of hemolymph than cutting a prolate pharate pupa, because the hemolymph volume is not lost. Individual hemolymph samples (0.8–1 mL/insect) were immediately mixed with 100% saturated ammonium sulfate (pH 7.0) to prevent the rapid melanization of hemolymph, which occurs at this developmental stage. The ammonium sulfate was adjusted to 50% saturation, and the suspension was stored at −70 °C. Hemolymph collected this way contains many active proteinases and is stable for at least 2 years. For the bacterial induction experiment, fifth instar M. sexta larvae (day 2) were injected with Micrococcus luteus (10 μL/larva, 10 μg/μL, Sigma), formalin-killed Escherichia coli XLI-blue (107 cells/larva, 10 μL/larva) (19), and NaCl (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM CaCl2, pH 7.8). One unit of activity is carried out at 4 °C for 5 min. After gel separation and transfer, the first six residues of the 25-kDa band were determined as described above.

Amino Acid Sequence Determination—The partially purified IEARase (PAP-2) was treated with SDS-sample buffer containing 2-mercaptoethanol and separated by electrophoresis on a 12% polyacrylamide gel (18). The protein was then transferred to a polyvinylidene difluoride membrane and stained with Amido Black (Sigma). The 35-kDa polypeptide, which corresponded to a [3H]DFP-labeled band detected as described by Jiang et al. (9), was subjected to automated Edman degradation. To determine the amino-terminal sequence of its 25-kDa light chain, purified PAP-2 (2 μg) was mixed with 1 μg of pyroglycamate aminopeptidase (Roche Molecular Biochemicals) in 100 μL of 100 mM sodium phosphate (pH 8.0) containing 10 mM EDTA, 5 mM dithiothreitol, 5% glycerol (v/v), and 1 mM p-aminobenzamidine. The reaction was carried out at 4 °C for 18 h followed by 25 °C for 4 h. The proteins in the mixture were precipitated by trichloroacetic acid at a final concentration of 10%. After centrifugation, the pellet was washed with cold anhydrous acetone and dissolved in 20 μL of the SDS sample buffer at 95 °C for 5 min. After gel separation and transfer, the first six residues of the 25-kDa band were determined as described above.

Molecular Cloning and Sequence Analysis of PAP-2—from Phage DNA (0.1 μg) isolated from a bacteriophage-transduced phage DNA library was used as a template in a PCR to amplify a PAP-2 cDNA fragment. Two degenerate primers were designed based on the amino-terminal sequence of PAP-2 catalytic domain: 660 (5′-ACA GCC ATC GAY CAR TAY CNG CCG 3′-3′) and 661 (5′-G CTG CCG CTG ATH GAR TAY CAY AA-3′), which encode TADYNYPW and LALLEYKHK, respectively. Primer 625 (5′-CAT GAG SGG RCC RCC SGA RTC NCC-3′) is the reverse complement of the sequence encoding GDSGGPLM, a highly conserved sequence around the active site serine residue in the chymotrypsin family of serine proteinases. In a first PCR, primer 660 was used with primer T7, which anneals with sequence in the cloning vector near the 3′-end of the inserted cDNA, under the following conditions: 94 °C, 30 s; 53 °C, 40 s; and 72 °C, 80 s for 30 cycles. The reaction mixture (10 μL) was used directly as a template for a PCR by using primers 661 and 625 under the same cycling conditions. After electrophoresis of the resulting products on a 1% agarose gel, a DNA band of the expected size (about 0.6 kb) was recovered and cloned into the pGem-T vector (Promega). After this clone was confirmed by DNA sequence analysis to encode PAP-2, the PCR-derived cDNA fragment was labeled with 32P and used as a probe to screen the induced M. sexta fat body cDNA library in zAP II (Stratagene) (19). Positive clones were purified to homogeneity and subcloned by in vivo excision of pBluescript phagemids. Nucleotide sequence analysis was carried out using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

Northern and Western Blot Analyses of PAP-2 and Protein Levels—Fat body and hemocyte total RNA samples were prepared according to Wang et al. (20), separated by electrophoresis in agarose gels containing formaldehyde, transferred to nitrocellulose, and hybridized with 32P-labeled PAP-2 cDNA. A duplicate blot was hybridized with a cDNA for M. sexta ribosomal protein S3 (21) as a loading control. To detect a possible change in expression upon bacterial challenge, Manduca larvae were injected with buffer, Escherichia coli, or M. luteus. Cell-free hemolymph samples collected at 24 h after injection were resolved by electrophoresis on an SDS-polyacrylamide gel (18). Immunoblot analysis was performed using the polyclonal antiserum to PAP-2 as the first antibody.2

Optimized Purification of PAP-2—All procedures for purification of PAP-2 were carried out at 4 °C. A frozen bar stage hemolymph sample (40 ml) was thawed, and the protein precipitate was collected by centrifugation at 12,000 × g for 25 min. The pellet was resuspended in 80 mL of HT buffer (pH 6.8, 10 mM potassium phosphate, 0.5 mM NaCl), supplemented with 0.001% 1-phenyl-2-thiourea and 0.5 mM p-amino- benzenesulfonic acid. To remove the β-l-glucan recognition proteins in the hemolymph (22), curdlan (0.2 g) was incubated with the protein solution with gentle agitation for 10 min on ice. The reaction mixture was centrifuged at 15,000 × g for 30 min to remove the flocculent materials including curdlan. Saturated ammonium sulfate solution was slowly added to the supernatant to a final saturation of 35%. After centrifugation at 15,000 × g for 30 min, the pellet was collected and dissolved in 20 mL of HT buffer. The fractionated plasma sample was dialyzed against the same buffer (1.0 liter for 8 h, twice), and the resulting particulate substances were removed by passing the sample through a syringe filter (0.45 μm, low protein binding, Fisher).

The protein solution was diluted with equal volume of H2O and applied to a hydroxyapatite column (2.5-mm inner diameter × 7 cm; Bio-Rad) equilibrated with 0.5 × HT buffer. Following a washing step with 60 mL of 0.5 × HT and 50 mL of 1 × HT buffers, bound proteins were eluted at 0.5 mM/min for 4 h with a linear gradient of 20–150 mM potassium phosphate (pH 6.8), 0.5 mM NaCl. Fractions were analyzed by immunoblotting and an IEARase assay. Active fractions containing PAP-2 were combined and precipitated with ammonium sulfate (60% saturation). The precipitate was collected by centrifugation at 15,000 × g for 30 min, dissolved in 3.0 mL, 20 mM Tris-HCl, 0.5 mM NaCl, pH 7.5 (S100 buffer), and then immediately applied to a Sephacryl S100-HR column (2.5-mm inner diameter × 100 cm; Amersham Bio- sciences) equilibrated with the same buffer. The flow-through fraction was eluted with S100 buffer at a flow rate of 0.7 mL/min and fractions were collected at 2.5 mL/tube after the first 100 mL.

Fractions containing PAP-2 were pooled and supplemented with CaCl2 and MgCl2 at a final concentration of 1 mM each. The sample was loaded onto a ConA-Sepharose 4B column (5.0 mL) equilibrated with 20 mM sodium acetate, 0.5 mM NaCl, 1 mg/mL sodium azide, 7.4 and washed with the same buffer until A280 was lower than 0.05.

The flow-through fraction was dialyzed against Q buffer (25 mM

2 C. Ji, Y. Wang, J. Ross, and H. Jiang, manuscript in preparation.
Tris-HCl, pH 7.5), supplemented with 0.5 mM p-aminophenazone (2 liters each time for 8 h, twice). After passing through a syringe filter, the dialyzed sample (45 ml) was applied to a UNO-Q6 column (6 ml; Bio-Rad) equilibrated by Q buffer. Following a washing step, the bound proteins were eluted with a gradient of 0–0.2 M NaCl in Q buffer at a flow rate of 1.0 ml/min for 30 min. This step was performed in a cold chamber using a Biologic Duo-Flow Protein Purification System (Bio-Rad). Fractions (0.5 ml/tube) were collected and analyzed by electrophoresis on a SDS-polyacrylamide gel (10%) followed by silver staining (23) or immunoblot analysis. Affinity labeling with [3H]DFP and fluorographic detection of PAP-2 were carried out according to Skinner and Griswold (24). Purified PAP-2 was stored at -70 °C for characterization and activity assays.

MALDI Mass Spectrometry of PAP-2 and Cleaved proPO—The purified PAP-2 was desalted using a C18 zip tip (Millipore) and eluted with 70% acetonitrile, 0.1% trichloroacetic acid. The sample was mixed with an equal volume of saturated sinapinic acid matrix on a MALDI plate, air-dried, and subjected to mass determination on a Voyager Elite mass spectrometer (PerkinElmer Life Sciences) with delayed extraction. The spectra were calibrated using bovine serum albumin as an external standard.

To determine the site at which PAP-2 cleaves proPO, 1.0 μg of proPO was incubated with 0.2 μg of PAP-2 on ice for 60 min, and the reaction mixture was desalted and eluted as discussed above. Molecular masses of peaks that were not present in the control spectra of proPO and PAP-2 were compared with calculated values of the amino-terminal peptides to determine the cleavage site in proPO.

Activation of proPO by PAP-2 in the Presence of Serine Protease Homologs (SPHs)—The SPH-1 and -2 were isolated from induced larval hemolymph according to Yu et al. (13). The protein preparation (10 μl) was incubated with 40 μl of 5 mM phenylmethylsulfonyl fluoride (Sigma) in 5 mM CuCl2, 25 mM Tris-HCl (pH 7.5) at 4 °C for 16 h to inactivate serine proteases that might be associated with the SPHs. The treated sample was dialyzed against the same buffer without phenylmethylsulfonyl fluoride (100 ml/8 h each time, twice). To test the effect of M. sexta SPH-1 and SPH-2 on the activation of proPO, purified proPO was incubated with buffer, PAP-2, SPH-1 and -2, or a mixture of PAP-2 and the SPHs at 4 °C for 60 min. PO activity in the reaction mixtures was assayed, using dopamine as a substrate. As described in the legend to Fig. 6, the reaction mixtures were also subjected to electrophoresis followed by silver staining or immunoblot analysis using antisem against Manduca proPO (diluted 1:2000) as the first antibody. To study whether PO activity is involved in the formation of SDS-stable PO polymers, we included 0.001% 1-phenyl-2-thiourea (PTU), a PO inhibitor, in some of the reaction mixtures and analyzed the samples similarly.

RESULTS

Partial Purification of PAP-2 and Its Requirement for a Co-factor—Preliminary experiments indicated that active PO and proteinases are already present in the hemolymph of bar stage prepupal M. sexta larvae in the absence of microbial challenge. As a step toward understanding the prophenoloxidase-activating system in M. sexta hemolymph, we attempted to purify a PAP from plasma of bar stage insects. To preserve the enzyme activities and minimize protein cross-linking caused by active PO, we kept the plasma and column fractions at high salt concentrations in the presence of inhibitors of phenoloxidase and proteases. Based on our previous experience with the cuticular PAP (PAP-1) (9), we employed three different assays to follow each purification step: proPO activation, [3H]DFP labeling, and an amidase assay using acetyl-Ile-Glu-Ala-Arg-p-nitroanilide as a substrate. Among the commercially available peptidyl-p-nitroanilide substrates, IEARpNa is most similar to the amino-terminal side of the putative activation site in M. sexta proPO (leu-ser-asn-arg-p-nitroanilide) as a substrate. Among the commercially available peptidyl-p-nitroanilide substrates, IEARpNa is most similar to the amino-terminal side of the putative activation site in M. sexta proPO-p1 (Leu-Ser-Asn-Arg) (25) and proPO-p2 (Leu-Asn-Asn-Arg) (25) (17). When the 15–40% ammonium sulfate fraction of the cell-free hemolymph was separated on a hydroxyapatite column, the proPO-activating activity roughly coincided with one of the IEARase peaks (data not shown). These fractions were pooled, concentrated, and then resolved by gel filtration chromatography on a Sephacryl S100-HR column (Fig. 1A). We detected a major IEARase activity peak in fractions 30–34 as well as low levels of IEARpNa hydrolysis in some other column fractions. This amidase peak did not correspond to the proPO-activating activity found in fractions 10–16 (data not shown). The proPO-activating activity in all of the fractions accounted for only 5–10% of the total activity loaded onto the column. One possibility is that, like PAP-1 (9), proPO activation is mediated by a complex of a proteinase and a protein cofactor, and separation of this complex led to the low activity of PAP-2.

To test this hypothesis, we incubated aliquots of fraction 32 with a small amount of other column fractions for 1 h along with purified proPO. Fraction 32 did not contain PO activity, and it generated little active PO when incubated with proPO (Fig. 1B). After fraction 32 was incubated with proPO in the presence of fraction 10–16, which contained only low levels of PO and proPO-activating activities, a large increase in PO activity was observed. This result supported the hypothesis that proPO activation requires PAP and a cofactor. Because high amidase activity was detected in fraction 32 but not in fraction 14, we concluded that the PAP is probably present in...
fraction 32 and that the cofactor is in fraction 14. Their molecular masses are estimated to be about 50 and 200–500 kDa, respectively, based on their elution volume from the Sepharyl S-100 column. The pooled fractions 30–34 from the gel filtration column were passed through a ConA-Sepharose column and a Jacalin-agarose column to remove some plasma glycoproteins. The flow-through fractions from the lectin affinity columns were concentrated and separated on an anion exchange HPLC column. A single small IERase peak was present in fractions 26–28, and a [3H]DFP labeling experiment indicated that these fractions contained a single radioactive band at 35 kDa (data not shown). As shown on an SDS-polyacrylamide gel stained with Coomassie Blue, this band was prominent and well separated from other proteins (Fig. 1C). This 35-kDa band and a 25-kDa band were shifted to a 48-kDa position under nonreducing conditions (see Fig. 5), which suggests that the proteinase, named PAP-2, is composed of a catalytic heavy chain (35 kDa) and a regulatory light chain (25 kDa) linked by a disulfide bond. The 29 residues of the 35-kDa polypeptide were determined to be Ile-Leu-Gly-Gly-Glu-Ala-Thr-Ala-Ile-Asp-Gln-Tyr-Pro-Trp-Leu-Ala-Leu-Ile-Thr-His-Lys-Leu-Ala-Glu-Ile-Lys-Leu-Met (Fig. 2). This sequence is similar to a region at the beginning of the catalytic domain in PPAE from the silkworm Bombyx mori (12). The amino terminus of the 25-kDa light chain starts with a pyro-Glu residue, since, after deblocking with pyroglyutamate aminopeptidase, the newly exposed sequence was determined to be Ala-(Cys)-Thr-Leu-Pro-Asn (Fig. 2).

**Molecular Cloning and Structural Features of PAP-2**—We designed two degenerate primers encoding part of the amino-terminal sequence of the 35-kDa polypeptide. Using these primers along with a vector-specific primer (T7) and a degenerate primer based on a conserved sequence around the active site serine, we obtained a 0.6-kb cDNA fragment in a nested polymerase chain reaction and cloned it into a pGem-T plasmid vector. Sequence analysis confirmed that the cDNA encoded a sequence starting with Leu-Ala-Leu-Ile-Glu-Tyr-Pro-Leu-Ala-Leu-Ile-Asp-Gln-Tyr-Pro-Trp-Leu-Ala-Leu-Ile-Thr-His-Lys-Leu-Ala-Glu-Ile-Lys-Leu-Met. The rest of the sequence is typical of a serine proteinase from the S1 family (25). Using the [32P]-labeled cDNA fragment as a probe, we screened a bacteria-induced M. sexta larval fat body cDNA library. Approximately 1.0 x 10^6 positives were found in the first round screening of 28,000 plaques, indicating that PAP-2 mRNA is abundant at 105 plaques, and residues determining the primary specificity of PAP-2 are labeled (\@). A polyadenylation signal (AATAAA, underlined) near the 3’-end of the coding region is probably used for generating a short form of the mRNA, which ends at nucleotide 1365 followed by a 19-nucleotide poly(A) tail.

**Fig. 2. Nucleotide and deduced amino acid sequence of M. sexta PAP-2**. Amino acid residues, shown in one-letter codes, are aligned with the first nucleotide of each codon. The secretion signal peptide is underlined. The double underlined sequence of the mature protein was confirmed by deducing the amino acid sequence starting with Ala-Ala-Lys (Fig. 1C). The proteolytic activation site is indicated (\@), and the double-underlined sequence after that was determined by Edman degradation. The catalytic residues at the active site are indicated with asterisks, and residues determining the primary specificity of PAP-2 are labeled (\@). A polyadenylation signal (AATAAA, underlined) near the 3’-end of the coding region is probably used for generating a short form of the mRNA, which ends at nucleotide 1365 followed by a 19-nucleotide poly(A) tail.
Purification of *M. sexta* PAP-2 from Bar Stage Hemolymph—

With the polyclonal antiserum available, we detected proPAP-2 in the hemolymph of naïve *M. sexta* larvae in the days just before pupation. At day 5 of the wandering stage, −10% of thezymogen was converted to active enzyme (PAP-2). Preliminary experiments indicated that almost all PAP-2 was present in the 0–35% ammonium sulfate fraction of the plasma sample and that a β-1,3-glucan recognition protein was one of the major contaminating proteins in several chromatographic steps in the original purification scheme. Based on these results, an efficient method was developed to purify PAP-2 from the bar stage hemolymph.

First, we incubated the 0–50% ammonium sulfate fraction of the plasma with curdlan in the presence of 0.5 M NaCl and 0.001% 1-phenyl-2-thiourea. Phenoloxidase activity was greatly reduced under these conditions, and β-1,3-glucan recognition protein bound to the insoluble curdlan. After curdlan and cell debris were removed by centrifugation, ammonium sulfate saturation of the supernatant was slowly adjusted to 35%. The protein precipitate was collected by centrifugation and dissolved in and dialyzed against the hydroxylapatite H buffer. Compared with the starting material, over 90% of total proteins were removed in this simple step (Table I). However, PAP-2 represented only a small portion of the total IEARpNa-hydrolyzing activity in the sample. PAP-2 (fractions 41–46) was hidden under a broad IEARase peak in fractions 38–52 (Fig. 4A). Guided by results from immunoblot analysis, we pooled fractions 41–46, which contained 90% of PAP-2 loaded. The specific activity was increased −9.4-fold by this step. The proteins in the combined fractions were collected by ammonium sulfate precipitation and then separated by gel filtration chromatography on a Sephacryl S100-HR column (Fig. 4B). Most of the PAP-2 eluted in fractions 37–44, whereas hydrolysis of IEARpNa was also detected in many other fractions. The last IEARase peak (fractions 52–60) may represent a proteinase that interacts with the column matrix, since its apparent molecular mass is only about 10 kDa, a value much smaller than the size of a typical serine proteinase without a regulatory domain (25 kDa).

After small amounts of contaminating proteins were removed from the combined fractions by lectin chromatography on ConA-Sepharose, the PAP-2 sample was dialyzed and separated by ion exchange chromatography on an HPLC Q column (Fig. 4C). A major protein peak eluted from the column at about 150 mM NaCl. Most impurities from the loaded protein sample were removed in this step, and the specific activity of PAP-2 increased about 90-fold (Table I). Although PAP-2 is present at a low concentration in bar stage hemolymph of *M. sexta*, we successfully purified the enzyme with an overall yield of 73% and an increase in specific activity of $6 \times 10^4$.

Properties of Purified *M. sexta* PAP-2—As indicated by SDS-PAGE (Fig. 5A), purified PAP-2 is near homogeneity with an apparent molecular mass of 48 kDa under nonreducing conditions. In the presence of 0.1 M dithiothreitol, the heavy and light chains of PAP-2 were separated as 35- and 25-kDa bands. Due to possible allelic variations and/or glycosylation differences, microheterogeneity was observed in both polypeptides. Immunoblot analysis (Fig. 5B) indicated that both polypeptide chains were recognized by the polyclonal antibodies, although the polyclonal antiserum appears to recognize the light chain better than the heavy chain. Affinity labeling of the active site serine residue showed that the catalytic domain of *M. sexta* PAP-2 is located in the 35-kDa chain, which is attached to the unlabeled light chain by a disulfide bond (Fig. 5C). The apparent molecular masses of both chains are greater than the
The masses calculated from sequence, suggesting that both of the polypeptides are glycosylated (Fig. 2).

We determined by MALDI mass spectrometry that the molecular mass of PAP-2 is 46,450 Da (Fig. 6), which is larger than the calculated molecular mass for PAP-2 (45,801 Da). The mass difference may result from glycosylation at Ser or Asn residues (Fig. 2). In agreement with the results of SDS-PAGE analysis (Fig. 5), we have also detected a shoulder peak at both MH and MH2 positions, which may correspond to a minor component in the PAP-2 preparation. Genetic variations and/or posttranslational modifications could be responsible for the 176-Da mass increase.

**Activation of proPO by M. sexta PAP-2 and SPHs**—To test whether purified PAP-2 can activate Manduca proPO, we incubated PAP-2 with proPO for 60 min and detected a low level of PO activity (Fig. 7A). Since the control reaction of proPO alone does not have any PO activity, Manduca PAP-2 must have converted a small amount of proPO to PO. However, the activation reaction was much more efficient in the presence of a cofactor.

We have isolated from *M. sexta* hemolymph two SPHs that bind to a C-type lectin, *M. sexta* immulectin-2 (13, 14). cDNA cloning indicates that both SPHs lack a Ser residue at the expected position of the active site. Neither protein, therefore, is expected to have a catalytic activity. However, they are similar in amino acid sequence to prophenoloxidase-activating factor (PPAF)-III from a coleopteran insect, *Holotrichia diomphalia* (27). PPAF-III is a serine proteinase-like protein that assists PPAF-I in the conversion of proPO to PO. The *M. sexta* SPHs and *H. diomphalia* PPAF-III contain a clip domain at their amino termini. Because of the sequence similarity, we tested whether the *M. sexta* SPHs can serve as cofactors of proPO.

**TABLE I**

| Purification step                  | Volume | Protein$^a$ | Concentration | Amount | PAP-2$^b$ | Concentration | Amount | % | fold |
|-----------------------------------|--------|-------------|---------------|--------|-----------|---------------|--------|---|------|
| Treated hemolymph                 | 40.0   | 27.0        | 1080          | mg/ml  | 0.41      | 16.4          | 100    | 1 |      |
| 0–35% ammonium sulfate fraction   | 45.5   | 1.40        | 63.7          | mg     | 0.34      | 15.5          | 95     | 16|
| Hydroxylapatite HT                | 3.0    | 2.05        | 6.2           | µg/ml  | 4.67      | 14.0          | 85     | 150|
| Sephacryl S100-HR                 | 20.0   | 0.065       | 1.3           | µg     | 0.63      | 12.6          | 77     | 638|
| ConA flow-through                 | 45.0   | 0.025       | 1.1           | µg     | 0.28      | 12.6          | 77     | 737|
| HPLC UNO-Q6                       | 1.0    | 0.012       | 0.012         | µg     | 12.0      | 12.0          | 73     | 6.6×10^4|

$^a$Protein concentrations were determined by a modified Bradford method using a commercial kit (Pierce). Bovine serum albumin was used as the standard.

$^b$Concentration of PAP-2 was estimated by measuring the band intensity of immunoblots using an Eastman Kodak Co. DC120 digital camera and Kodak Digital Science 1D gel analysis software. Purified PAP-2 of known concentration was used as the standard in the immunoblot analysis.
PAP-2. In the control reactions, proPO and SPHs did not have PO activity (Fig. 7A). After proPO was incubated with PAP-2 and SPHs, however, we detected phenoloxidase activity at a level much higher than the control reactions of proPO with either PAP-2 or SPHs. These results indicate that the SPHs are required for efficient activation of proPO by PAP-2.

To explore the mechanism of proPO activation, we separated the reaction mixtures by electrophoresis on an SDS-polyacrylamide gel followed by either silver staining (Fig. 7B) or immunoblot analysis (Fig. 7C). M. sexta proPO is composed of 79-kDa proPO-p1 and 80-kDa proPO-p2 (17). After proPO had been incubated with PAP-2 for 1 h, a 74-kDa protein was generated, which was recognized by antibodies against proPO-p2 (data not shown). Therefore, we concluded that a significant amount of proPO-p2 was converted to a 74-kDa product, although a low level of PO activity was detected (Fig. 7A). In contrast, the cleavage of proPO-p1 was hardly detected. When proPO was reacted with PAP-2 in the presence of the SPHs, the 74-kDa band became more prominent. In addition, several other bands were detected: one at about 140 kDa and two at greater than 250 kDa (Fig. 7B), which were recognized by antibodies against Manduca proPO (Fig. 7C). These may be protein complexes that contain PO or PO oligomers. The protein complexes appear to be connected through covalent bonds, since they are present after the reaction mixture was separated by SDS-polyacrylamide gel electrophoresis under reducing conditions.

To test whether active phenoloxidase itself is required for the oligomer formation, we included a PO inhibitor (PTU) in the activation mixture. While the conversion of proPO-p2 to its 74-kDa form was not affected, we did not detect any high molecular weight complex in the presence of PO inhibitor (Fig. 7B and C). Thus, active PO may be involved in formation of covalent cross-links between proteins to form the observed high molecular weight species.

**Determination of Cleavage Site in proPO**—To further characterize the proPO activation reaction, we used MALDI mass spectrometry to determine the site at which PAP-2 cleaves proPO (Fig. 8). In the control samples of PAP-2 or proPO only, we did not detect any significant mass peak within the range from 2500 to 6500 Da (data not shown). However, after Manduca proPO was incubated with PAP-2, we detected a major peak at 5851 Da and a corresponding MH1 peak of 2926 Da. There was also a small peak of 6032 Da in the spectrum. These peaks probably correspond to the propeptides released from proPO-p1 and proPO-p2 as a result of proteolytic cleavage by PAP-2. Previous study indicated that proPO-p1 and
proPO-p2 are blocked at the amino terminus (17). We calculated the molecular masses for the putative 50-residue propeptides to be 5990 Da for proPO-p1 and 5808 Da for proPO-p2. Taking into account the accuracy of MALDI time-of-flight mass spectrometry, the mass differences between the theoretical and observed mass values (42 Da for propeptide 1 and 43 Da for propeptide 2) are in good agreement with \( \text{N} \)-acetylation of the amino-terminal residue. The silkworm proPO-p1 and proPO-p2, cleaved by PPAE at Arg51, were also found to be \( \text{N} \)-acetylated (28).

**DISCUSSION**

As an integral part of the host defense system against invading pathogens and parasites, prophenoloxidase activation has been investigated in various insects and crustaceans for more than 30 years (3, 4). Even so, our knowledge of the prophenoloxidase-activating cascade is rather limited. A major breakthrough currently occurring in this field is the isolation and molecular cloning of clip domain serine proteinases that directly activate prophenoloxidase through limited proteolysis (9, 12, 30, 31). We previously isolated PAP-1 from *M. sexta* and cloned a cDNA encoding the proPAP-1 zymogen (9). PAP-1 contains a single amino-terminal clip domain. proPO-activating proteinases from the beetle *H. diomphalia* (30) and from a crayfish *Pacifastacus leniusculus* (31) also contain one clip domain. In this paper, we describe a second PAP from *M. sexta*, PAP-2. PAP-2 is most similar to the silkworm PPAE (12). *M. sexta* PAP-2 and silkworm PPAE contain two amino-terminal clip domains with 24 cysteine residues that are perfectly aligned. The functions of clip domains in arthropod serine proteinases are not yet well established. It seems likely that proPO-activating proteinase-2 from *M. sexta* Hemolymph

**FIG. 7.** Effect of *M. sexta* SPH-1 and SPH-2 on the activation of prophenoloxidase by PAP-2. A, PO activity. Buffer, SPHs (5 ng), PAP-2 (50 ng), or a mixture of PAP-2 and SPHs (50 ng + 5 ng) was incubated with 0.1 \( \mu \)g of purified proPO in 20 mM Tris-HCl, 5 mM CaCl\(_2\), pH 7.5, at 4 °C for 60 min. Activated phenoloxidase was assayed using dopamine as a substrate. B, SDS-polyacrylamide gel electrophoretic analysis. Purified proPO (0.125 \( \mu \)g) was reacted with buffer (lanes 1 and 4), 20 ng of PAP-2 (lanes 2 and 5), or a mixture of 20 ng of PAP-2 and 7.5 ng of SPHs (lanes 3 and 6) in the absence (lanes 1–3) or presence (lanes 4–6) of 0.001% PTU. The reaction mixtures were treated with SDS-sample buffer containing \( \beta \)-mercaptoethanol, separated by electrophoresis on a 6% polyacrylamide gel, and visualized by silver staining. C, immunoblot analysis. Purified proPO (0.1 \( \mu \)g) was incubated with buffer (lane 1) or a mixture of 50 ng of PAP-2 and 30 ng of SPHs in the absence (lane 2) or presence (lane 3) of PTU. The activation mixtures were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Immunoblot analysis was performed using 1:2000 diluted antiserum against proPO as the first antibody. Positions of the prestained molecular weight standards are indicated on the right.

**FIG. 8.** MALDI time-of-flight mass spectrometry of propeptides released from proPO after PAP-2 cleavage. The reaction mixture of proPO and PAP-2 was desalted and eluted from a C18 zip tip. A representative strong single accumulation spectrum is presented. The spectrum was subjected to noise removal and calibrated with external standards of bovine insulin, *E. coli* thioredoxin, and horse apomyoglobin. The mass values are indicated above the MH\(^+\) and MH\(^{2+}\) peaks. Other experimental details are described under “Experimental Procedures.”
they are involved in regulating the enzymes’ activity or localization. The clip domain in the crayfish proteinase exhibits antibacterial activity toward Gram-positive bacteria (31). If clip domains interact with bacteria to exert an antimicrobial function, they might also anchor the PAP-2 catalytic domain that converts proPO to PO onto a bacterial surface. Clip domains linked to serine proteinases may have multiple functions in arthropod immune responses (10).

The two PAPs we have identified in *M. sexta* have some similarities and some differences in their patterns of expression. *M. sexta* PAP-1 was initially isolated from a prepupal cuticular extract, and its mRNA is present in prepupal epidermis. PAP-1 mRNA was also detected in fat body of naïve (no microbial injection) feeding stage larvae, and proPAP-1 is present in hemolymph of naïve larvae (32). Upon bacterial challenge, the concentration of proPAP-1 protein increases in hemolymph, as transcription of the PAP-1 gene is up-regulated in fat body and hemocytes during immune responses (9, 32). However, neither proPAP-2 nor its mRNA was detected in naïve feeding stage larvae (Fig. 3). During the larval stage, PAP-2 expression was only observed after microbial challenge. However, we did detect proPAP-2 and active PAP-2 in hemolymph of naïve prepupal insects, but PAP-1 was not present in hemolymph at this stage. These results suggest that the transcription of the PAP genes might be regulated by developmental and immune pathways. Hormonal signals (presumably ecdysteroids) prior to pupation may stimulate expression of PAP-1 in immune pathways. Hormonal signals (presumably ecdysteroids) prior to pupation may stimulate expression of PAP-1 in immune pathways.

Purified *M. sexta* PAP-1 and PAP-2 require the presence of a nonproteolytic protein cofactor for proPO-activating function, although they can hydrolyze a small peptide substrate in the absence of the cofactor (Fig. 1). The cofactors responsible for this modulation of PAP activity appear to be SPHs, which have a structure similar to the PAPs themselves. These SPHs contain an amino-terminal clip domain and a carboxyl-terminal proteinase-like domain, but their active site serine residue is replaced with glycine (13). The SPHs isolated from *M. sexta* plasma are a mixture of two related proteins, which we have not yet been able to separate. We are pursuing experiments with recombinant SPHs to determine whether one or both of the SPHs interact with PAP-1 and PAP-2.

Similar to *M. sexta* PAP-1 and PAP-2, PPAF-I from a beetle, *H. diomphalia*, was also reported to require a proteinaceous cofactor for proPO-activating function, in contrast to the proteinases from *M. sexta* PPAF-II was also present, proPO was cleaved at an additional site in proPO after incubation with PAP-2 and SPHs (Fig. 7). In contrast to the proteinases from *M. sexta* and *H. diomphalia*, the silkworm PPAE, which contains two clip domains like PAP-2, does not appear to require any protein cofactor for the activation (12). The reason for this difference is not clear. Perhaps *in vivo* at a low enzyme concentration, the silkworm PPAE may also interact with SPH or other protein cofactors.

Nonself recognition, an essential first step of the insect-immune responses against microorganisms, is mediated by hemolymph proteins that specifically bind to molecules present on the surface of invading pathogens, such as lipopolysaccharide and peptidoglycan from bacteria or glucans and mannans from fungi (33, 34). Lectins involved in proPO activation (14, 35) and some cellular defense responses (36) have been identified in several insect species. *M. sexta* immunie-2, a C-type lectin, binds to bacterial lipopolysaccharide, interacts with the SPHs, and stimulates proPO activation in response to lipopolysaccharide (14, 15).

It can be envisioned that bacterial infection results in formation of a protein complex, containing immunie-2 bound to the bacterial surface and interacting with SPHs, which bring PAP and proPO together in a suitable orientation for activation of proPO by PAP-1 or PAP-2 (13). Such a complex serves the purpose of activating proPO and localizing the active enzyme at a microbial surface. We observed that activation of proPO by PAP-2 in the presence of SPHs resulted in the formation of high molecular weight species that contained PO (Fig. 7) but not PAP-2 or SPH. Therefore, we interpret these bands, detected in SDS-PAGE and immuno blot analysis, to represent covalently linked oligomers of PO or PO associated with other proteins, because they were not dissociated by 1% SDS. Formation of the oligomers did not occur in the presence of a PO inhibitor, indicating that the PO enzymatic activity is required to cross-link the proteins, perhaps through oxidation of tyrosine residues. Beck et al. observed a high molecular weight protein complex, including proPO and PO, that formed in *M. sexta* plasma only when the hemolymph was collected under nonsterile conditions, suggesting that it was a response to the presence of microorganisms. Formation of a protein complex at a surface is a common feature of serine proteinase cascade pathways such as the vertebrate blood coagulation and complement systems. A similar process may occur in arthropod proPO activation cascades, serving to activate proteinases in discrete locations near the surface of pathogens. We are planning to express the PAPs, SPHs, and their individual clip and proteinase domains as recombinant proteins for further studies to understand the interactions among the protein factors involved in proPO activation in *M. sexta*.

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Prophenoloxidase-activating Proteinase-2 from Hemolymph of *Manduca sexta*: A BACTERIA-INDUCIBLE SERINE PROTEINASE CONTAINING TWO CLIP DOMAINS

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