Prophenoloxidase-activating Enzyme of the Silkworm, Bombyx mori

PURIFICATION, CHARACTERIZATION, AND cDNA CLONING

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Prophenoloxidase-activating enzyme (PPAE) was purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis from larval cuticles of the silkworm, Bombyx mori. The purified PPAE preparation was shown to be a mixture of the isozymes of PPAE (PPAE-I and PPAE-II), which were eluted at different retention times in reversed-phase high performance liquid chromatography. PPAE-I and PPAE-II seemed to be posttranslationally modified isozymes and/or allelic variants. Both PPAE isozymes were proteins composed of two polypeptides (heavy and light chains) that are linked by disulfide linkage(s) and glycosylated serine proteases. The results of cDNA cloning, peptide mapping, and amino acid sequencing of PPAE revealed that PPAE is synthesized as prepro-PPAE with 441 amino acid residues and is activated from pro-PPAE by cleavage of a peptide bond between Lys152 and Ile153. The homology search showed 36.9% identity of PPAE to homologous to the arthropod hemocyanins, we have previously speculated that the pro-PO may be involved in physiological processes other than defense mechanisms, such as O2 transport (10).

Another serine proteinase cascade in insects is involved in the establishment of the dorso-ventral pattern in the Drosophila embryo (12). Very recently, Lemaître et al. (13) have provided evidence demonstrating the links between the dorso-ventral pattern formation and immune protein induction. They reported the involvement of components of the dorso-ventral pattern formation pathway, named spätzle and Toll, in the induction of an antifungal peptide (drosomycin) synthesis in the fruit fly, Drosophila melanogaster, following fungal infection. At the blastoderm stage of the Drosophila embryo, spätzle is a ligand of the cell surface receptor, Toll, and is produced from pro-spätzle following a limited proteolysis by the serine protease easter in the perivitelline space. The binding of spätzle to Toll causes activation of the intracellular signaling pathway for the dorso-ventral pattern formation. These previously reported observations raise the possibility that easter is involved in the extracellular signaling pathways for drosomycin synthesis and the dorso-ventral pattern formation in Drosophila. The parallels between intracellular signaling pathways and the synthesis of immune proteins with antimicrobial activity effectively engage in defending the insect from the invasion of microorganisms (1–3).
ways for the dorso-ventral pattern formation of the Drosophila embryo and activation of the Drosophila immune protein gene have been known for several years (14). Thus, the links between the dorso-ventral pattern formation and immune protein induction seem to be much closer than they had previously been thought to be.

Before the implication of the Toll pathway, the pro-PO cascade was the only protease cascade known to be present in larval and adult stages of insects. At present, two serine proteases of the insect pro-PO cascade have been purified. One of them, which is tentatively referred to as BAEase, was purified from the silkworm hemolymph and has been shown to be activated from its zymogen (pro-BAEase) by limited proteolysis (15). The function of pro-BAEase remains unknown. The other enzyme, pro-PO-activating enzyme (PPAE), was purified by Dohke 26 years ago (16) from larval cuticles of silkworms. We previously reported that PPAE is also present as an inactive zymogen (pro-PPAE) in the hemolymph and cuticles of silkworm larvae (6, 10). Since the first purification by Dohke, PPAE has been purified from several insect species (17–20). However, characterization of PPAE at the molecular level and elucidation of the activation mechanism of PPAE zymogen remain to be carried out.

Here, we report the purification, characterization, and cDNA cloning of PPAE. PPAE is shown to be a homologous protein to easter and to be synthesized in the epidermis of the integument, the hemocytes, and the salivary glands in the larval stage of the silkworm, Bombyx mori. Preliminary immunological study suggests the presence of pro-PPAE in silkworm eggs.

**EXPERIMENTAL PROCEDURES**

**Silkworm (B. mori).**—For obtaining cuticles, silkworms at the beginning of the fifth instar were purchased from local sericulture farms. They were reared on mulberry leaves at room temperature (22–28 °C) to day 5 or 6 of the fifth instar. Cuticles were collected according to the method of Dohke (16) and stored at −20 °C until use. For experiments other than the purification of PPAE, silkworms were reared on an artificial diet as described previously (6). Nondiapause eggs laid within 2 h were collected, and all of them were designated to be at 1 h from the oviposition.

**Preparation of CH-Sepharose 4B Coupled to m-Aminobenzamidine—**4 g of m-aminobenzamidine dihydrochloride (Sigma) was coupled to 15 g of CH-Sepharose 4B by using 5 g of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) at pH 4.75 according to the manufacturer's instructions. The resulting CH-Sepharose 4B coupled to m-aminobenzamidine (Sepharose-mABA) was subjected to eight alternate washings with 10 mM HCl containing 0.5 mM NaCl and with 10 mM NaOH containing 0.5 mM NaCl, followed by final washing with 1 liter of distilled water.

**Pro-PO and the Assay of PO Activity—**Pro-PO was purified from hemolymph of the silkworm, B. mori, as described previously (21), and the amount of one unit of PO was defined according to Ashida (22).

**Assay of PPAE—**PPAE was assayed using the following three different substrates: (a) A mixture of equal volumes of pro-PO (0.180 mg/ml of 10 mM potassium phosphate buffer, pH 7.5) and PPAE was incubated on ice, and an aliquot of the mixture was assayed for PO activity at 5 and 15 min from the beginning of the incubation using 1-DOPA as a substrate, according to the method of Ashida (22). During assay of PO activity, the activation of pro-PO was insignificant because pro-PO and PPAE were diluted 50-fold in the assay mixture, and pH was lowered to 6.0. One unit of PPAE was defined as the amount that produced 200 units of PO in 5 min of PO from 5 to 15 min of the incubation. In the activation reaction, the concentrations of the buffer solution and the PPAE were 0.01 M and below 2.5 units/ml, respectively, and pH was maintained at 7.5. This assay method was used in the course of purification of PPAE. (b) The standard assay mixture for determining esterase activity was composed of 1 mM BAEE, 50 mM Tris-HCl buffer, pH 8.0, at 25 °C, 0.5 mM KCl and enzyme in a final volume of 1 ml. The reaction was initiated by the addition of enzyme at 25 °C and monitored at 254 nm using a Shimadzu spectrophotometer (model 240) with a constant temperature cuvette holder and an accessory unit (OHI-4) for memorizing the monitored data and processing them to output in a form of the differential. In the experiments to determine $K_m$ and $V_{max}$ at varied KCl concentrations, the concentrations of BAEE at certain times were read on the records of the time course of the reactions, and the values of the differential of the time courses were read at the corresponding times. Thus, the velocities of the hydrolysis at many different concentrations of BAEE were obtained at the same time and the time course of the reaction with high accuracy. The activity was expressed as μmol substrate hydrolyzed/min/mg protein under the above conditions. The concentration of BAEE hydrolyzed was calculated from the molar absorbancy index adopted from Schwert and Takenaka (23). (c) Amidase activity of PPAE was assayed by using various fluorogenic substrates, peptidyl-7-amino-4-methyl coumarins (NH-Mecs, Peptide Institute Inc.). The reaction mixture was composed of 20 mM Tris-HCl buffer, pH 7.5, at 25 °C, 0.2 mM NaCl, 50 μM NH-Mecs, and PPAE solution in a total volume of 0.5 ml. After incubation of the reaction mixture at 25 °C for 60 min, the enzyme reaction was terminated by adding 0.5 ml of 50% (by volume) acetic acid. The amount of liberated 7-amino-4 methyl coumarin was determined by the method of Kojima et al. (24).

**Preparation of Ammonium Sulfate Fractions of PPAE from Cuticles of Silkworm Larvae—**Ammonium sulfate fractions of PPAE were prepared by two methods as described below. All subsequent procedures were performed at 0–4 °C, and centrifugation was carried out at 12,000 × g for 20 min unless otherwise specified. pH values of buffers were those at room temperature (22–26 °C). (a) 4 kg of frozen cuticles obtained from 15-10 silkworms were extracted with ammonium sulfate, fractionated with ammonium sulfate, and dialyzed according to the method of Dohke (16). The resultant fraction was designated PPAE (AS-dw). The PPAE (AS-dw) was 436 ml in volume and contained 181,314 units of PPAE and 13.42 g of protein. Its specific activity was 13.46 units/mg protein. (b) The residue of cuticles extracted with distilled water in a above was homogenized in a Warning blender (model 34BL21) for 30 s at the maximum speed in 4 liters of 10 mM Tris-HCl buffer, pH 7.1, containing 20 mM CaCl2 and 0.5 mM NaCl. The homogenate was stirred for 2 h, followed by centrifugation. The supernatant was subjected to ammonium sulfate fractionation as in a, and the precipitate that appeared between 0.2 and 0.4 saturation was dissolved in 660 ml of Tris-HCl buffer, pH 8.1, containing 10 mM CaCl2 and dialyzed against 10 liters of the same buffer for 24 h with two changes of the buffer. The dialyzed solution was centrifuged at 77,000 × g for 30 min, and the supernatant was named PPAE (AS-salt). The PPAE (AS-salt) was 1.145 ml in volume and contained 2,999,900 units of PPAE and 11.7 g of protein. Its specific activity was 256.9 units/mg protein.

**Purification of PPAE from PPAE (AS-salt)—**Column chromatography of PPAE on DEAE-cellulose (DE23, Whatmann), hydroxyapatite (Shandon, UK), and CM-cellulose (CM52, Whatmann) was carried out by adopting essentially the same methods as those described by Dohke (16). Briefly, PPAE (AS-salt) was applied to a DEAE-cellulose column (7.0 × 55 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.5. Unabsorbed materials were washed with the same buffer at a flow rate of 200 ml/h. 20-ml fractions were collected from the beginning of the sample application. Fractions containing PPAE were pooled. PPAE fraction containing 50% saturation of KCl was dialyzed against 50% KCl, and the PPAE fraction was applied to a hydroxyapatite column (3 × 30 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.5, followed by washing of the column with 300 ml of the buffer. Adsorbed proteins were eluted at a flow rate of 50 ml/h with a linear potassium phosphate gradient from 0.01 to 0.5 M at pH 7.5 in a total volume of 1 liter. 10-ml fractions were collected from the beginning of the gradient elution. Active fractions (numbers 37–48, first HA fraction) were dialyzed against 5 liters of 10 mM potassium phosphate buffer, pH 7.5, for 12 h. The dialyzed fractions were loaded onto a CM-cellulose column (1.8 × 30 cm) previously equilibrated with the same buffer. After washing the column with 100 ml of the buffer, a linear gradient of KCl (0–0.4 M) in 600 ml of the buffer was applied to the column at a flow rate of 25 ml/h, and 6-ml fractions were collected from the beginning of the gradient elution. Active fractions (numbers 1–20, CM fraction) were pooled and subjected to further purification. The CM fraction was loaded onto a column (1.5 × 11.5 cm) of Sepharose-MABA after adjustments of the NaCl concentration to 0.5 M and pH to 8.0 by adding 0.66 mM NaCl and 1 mM Tris solution, respectively. The column was eluted sequentially with solutions of 10 mM Tris-HCl buffer, pH 8.0, containing the following volumes of NaCl (the volume of the solution is indicated in parentheses): 0.5 mM (500 ml), 0.1 mM (100 ml), 0.05 mM (300 ml), and 0 mM (150 ml). 6-ml fractions were collected from the beginning of the elution with the buffer containing 0.05 mM KCl and fractions (numbers 4–52) were pooled (Sepharose-MABA fraction). After pH of the Sepharose-MABA fraction was adjusted to 6.5 by the addition of 0.5 ml of 1 M potassium phosphate buffer and about 33 ml of
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0.01 N HCl, the fraction was applied to a hydroxypatite column (1.7 × 26 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 6.5. Adsorbed proteins were eluted at a flow rate of 20 ml/h with a linear gradient of potassium phosphate buffer, pH 6.5, (0.01–0.3 M) in a total volume of 240 ml. 3-ml fractions were collected from the beginning of the breakthrough fractions (number 100) corresponding to the gel, the mem-brane was cut into small pieces with PPAE-I and PPAE-II, and each of the pieces was blotted with one of the peroxidase-conjugated lectins (Peroxidase-conjugated Lectin kit-B, Seikagaku Kogyo) at a concentra-tion of 10 µg conjugated lectin/ml. The blots were visualized by incu-bation with 4-chloro-1-naphthol and H₂O₂. After the transfer of PPAE-I and PPAE-II onto the polyvinylidene difluoride membrane (Immobilon, Millipore), the mem-

Examination of pH Stability of PPAE. —One volume of PPAE (1.647 mg protein/ml of 10 mM potassium phosphate buffer, pH 6.5, containing 1% SDS, followed by 15 volumes of buffer solutions with various pH values. In the buffer solutions (200 µl) containing PPAE, the concentration of the buffers was 71.4 mM and the KCl concentration was 71.4 mM. The diluted PPAE mixtures were incubated on ice for 24 h, followed by the addition of 15 volumes of 1 M Tris-HCl buffer, pH 8.0. After the dilution, an aliquot (15 µl) of each PPAE solution was assayed for activity by using BAEF.

SDDS-PAGE, PAGE under Nondenaturing Conditions, and Isoelectric Focusing PAGE—SDDS-PAGE was carried out in a 1-mm-thick slab gel according to the method of Laemmli (26), with 12% acrylamide in the separation gel unless otherwise specified. PAGE under nondenaturing conditions was performed in 7.5% separating gel at pH 9.2 and 6.5 according to the method of Davis (27).

Isoelectric focusing-PAGE was performed according to the method of Wyckoff (28), containing 5.0% acrylamide, 0.25% bisacyr-amide, and 2% Ampholine, pH 3.5–10 (Amersham Pharmacia Biotech) was prepared in a column (inner diameter, 110 × 2.5 mm). After 5 µg of PPAE had been electrofocused for 12 h at 200 V and then for 1 h at 400 V, the gel was treated with 12% trichloroacetic acid and then stained for protein with Coomassie Brilliant Blue R-250 (CBB). The gel was calibrated with the following isoelectric point markers (Amersham Pharmacia Biotech): amyloglucosidase (pI 3.50), glucose oxidase (pI 4.15), soybean trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and pI 7.35), lentil lectin (pI 8.15, pI 8.45, and pI 8.65), and trypsinogen (pI 9.30).

Samples Used in SDDS-PAGE and Immunoblotting—The plasma frac- tion of hemolymph was obtained as described previously (6). Extracts of cuticles were prepared by using 5% SDS and 5 M urea (10) in the presence or absence of 3% 2-mercaptoethanol. Eggs (100 mg) and ovaries including oviducts (300 mg) were homogenized in 300 µl of 10 mM potassium phosphate buffer, pH 7.5, containing 1% SDS, followed by centrifugation at 15,000 × g for 30 s. After the supernatants were subjected to centrifugation again under the same conditions, the resulting supernatants were used as the extracts.

Gel Permeation Chromatography of PPAE on Sephacryl S-200—A Sephacryl S-200 (Amersham Pharmacia Biotech) column (1.08 × 100 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl. The column was calibrated by chromatography of a mixture of molecular mass standards (Combthek Calibration Proteins II, Roche Diagnostics): bovine α-chymotrypsin (25 kDa), hen egg albumin (45 kDa), myoglobin (16.9 kDa), bovine carbonic anhydrase B (5.85 kDa), human carbonic anhydrase B (6.55 kDa), horse myoglobin (6.85 and 7.35), lentil lectin (8.15, 8.45, and 8.65), and trypsinogen (9.30).

Mass Number Analysis by Matrix-assisted Laser Desorption and Ionization (MALDI) Spectrometry —Mass number analyses were carried out according to the method of Peter et al. (29) by using a Kompact MALDI 4 (Shimadzu Corp.) operated in linear time of flight mode. Ionization was accomplished by nitrogen laser at a wavelength of 337 nm for 3 ns. Sinapinic acid was used as a matrix. A saturated matrix solution was prepared in 0.1% trifluoroacetic acid containing 50% ace-tonitrile (AcCN). After the saturated matrix solution was dried on a C₈ column, and adsorbed polypeptides were eluted with a linear gradient of AcCN in 0.1% trifluoroacetic acid (5–65% AcCN in 65 and 65–100% AcCN in 15 min) at a flow rate of 0.5 ml/min. The light chain (PPAE-I-L) and heavy chain (PPAE-I-H) of PPAE-I were eluted at about 17.1 and 27.5 min, respectively, from the beginning of the gradient. The S-S-pyridylethylated light chain (PPAE-II-L) was eluted at the same retention time as that of PPAE-I, but the heavy chain (PPAE-II-H) of PPAE-II was eluted at 28.6 min. The S-S-pyridylated light chains and heavy chains were lyophilized.

Peptide Mapping of the S-S-Pyridylethylated Light Chain and Heavy Chain of PPAE-I—The light chain (PPAE-I-L) and heavy chain (PPAE-I-H) of PPAE-I obtained from 5 nmol of PPAE-I were digested with lysyl endopeptidase (Wako Pure Chemical Industries) at a molar ratio of 100:1 in 0.1 M Tris-HCl buffer, pH 8.5, containing 4 M urea in a total volume of 320 µl at 37 °C for 24 h. The peptides in the digests of the S-S-pyridylethylated PPAE-I-H and the PPAE-I-L were separated by reversed-phase HPLC on an ODS column (YMC-Pack ODS-AP; pore size, 300 Å; column size, 4.6 × 250 mm). The former was eluted by an AcCN gradient (10–100% AcCN in 0.1% trifluoroacetic acid in 90 min), and the latter was eluted by consecutive gradients (0–60% AcCN in 0.1% trifluoroacetic acid in 60 min and 60–100% AcCN in 0.1% trifluoroacetic acid in 10 min) at a flow rate of 0.8 ml/min. Peptides eluted in well separated peaks were analyzed for their amino acid sequences.

Amino Acid Sequence Analyses—Amino acid sequences were exam-ined according to the method of Edman and Beggs (30) on a protein sequencer PPSQ-10 (Shimadzu Corp.).

Deblocking of N-terminal Amino Acid of PPAE-I-L and PPAE-II-L—PPAE-I-L and PPAE-II-L were deblocked by Pfu pyrogulutaminate ampi-nopetidase (Takara). Approximately 4.2 nmol of lyophilized S-pyridylethylated PPAE-I-L or PPAE-II-L were digested with Pfu (25 units) in 250 µl of 50 mM sodium phosphate buffer, pH 7.0, containing 0.72 M guanidine-HCl, 10 mM dithiothreitol, and 1 mM EDTA at 50 °C for 6 h. The resulting samples were applied separately to a C₈ column and eluted under the same conditions as those for the separation of S-S-pyridylethylated light and heavy chains of PPAE. Both of the deblocked PPAE-I-L and PPAE-II-L were eluted as a single peak at about 28 min from the beginning of the gradient. The deblocked PPAE-I-L and PPAE-II-L were subjected to amino acid sequencing.

Labeling of PPAE with [3H]DFP and Fluorography of the Labelled Enzyme—Approximately 66 µg of PPAE was incubated with 30 n mole of [3H]DFP (222 Gb/mmol; NEN Life Science Products) in 356 µl of 10 mM potassium phosphate buffer, pH 7.5, containing 10 mM KCl for 12 h on ice. Labeled PPAE-I and PPAE-II were separated by a reversed-phase ODS column as is described in the legend to Fig. 2. The labeled PPAE-I and PPAE-II eluted from the column were lyophilized.

For fluorography, each of the labeled PPAE-I and PPAE-II was dissolved in 53 µl of 8 M urea. A half volume of each dissolved solution was run on SDDS-PAGE (26) under reducing conditions and the other half under nonreducing conditions. 2-mm-thick and 17% polyacrylam-ide-separating gel was employed in the SDDS-PAGE. After the elec-trophoresis, the gel was processed for fluorography by using Enlightning (NEN Life Science Products) according to the manufacturer’s instruc-tions and exposed to x-ray film (X-OMAT, Kodak) at −80 °C for 1 week before development.

Polycatonic Rabbit Anti-PPAE IgG—PPAE (320 µg) in phosphate-buffered saline was emulsified with an equal volume of Freund’s com-plete adjuvant and injected subcutaneously into a rabbit. Two addi-tional injections were administered at 10-day intervals. Blood was collected at 10 days after the last injection.

Anti-PPAE/IgG from the immunized rabbit serum and nonimmu-nized IgG from nonimmunized rabbit serum were obtained by protein A-Sepharose 4B (Amersham Pharmacia Biotech) column chromatogra-phy according to the manufacturer’s instructions.
**Immunoblotting**—After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane (31). Following the transfer, polypeptides cross-reactive to anti-PPAE/IGG were blotted by the standard method (32). Anti-PPAE/IGG and anti-rabbit goat IgG conjugated to horse radish peroxidase (Santa Cruz Biotechnology) were used at concentrations of 9.6 μg/ml and 1 μg/ml, respectively. The blots were visualized by incubation with H2O2 and 4-chloro-1-naphtol.

**Extraction of RNA**—On day 3 of the fifth instar, the silkworms were dissected in a dissecting buffer containing 0.11M KCl, 4 mM NaCl, 5 mM KH2PO4, and 4 mM EDTA that had been adjusted to pH 6.5 by KOH. The fat body, mid gut, integument, and silk gland were obtained from a larva, and salivary glands were obtained from 60 larvae. For collection of hemocytes, 60 larvae were bled by cutting one of their abdominal legs, and the discharged hemolymph was collected into 40 ml of the dissecting buffer that was gently being stirred. The collected hemolymph was centrifuged at 1,000 × g for 5 min at 4 °C, and the resulting hemocytes were suspended in 40 ml of the dissecting buffer. The suspension-and-centrifugation was repeated twice. The final sediment was used as hemocytes. Total RNA of the tissues was extracted using Isogen (Nippongene) according to the manufacturer’s instructions.

**Construction of cDNA Library of Larval Integument**—The mRNA in the total RNA obtained from larval integument was purified by the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). The integument cDNA library was constructed using the Lambda Zap II cDNA Synthesis Kit (Stratagene) and Gigapack III Goldpack Extract (Stratagene). Purification of the mRNA and construction of the cDNA library were performed according to the manufacturer’s instructions.

**PCR and Subcloning of the PCR Product**—The degenerate primers, 5'-AAGAATTTCTA/CT/CA/AG/CA/A/G/C/A/G/C/CT/TA/CT/CA/CT/TT/T-3', and 5'-AAGAATTTCCCA/A/C/CT/CC/CA/GT/AG/GT/A/G/CT/A/C-G/T/T/CTA/CT/TA/-3', were synthesized on the basis of the sequence of peptides of YQPYHF and YEYLPW, respectively, which were obtained from lysyl endopeptidase digest of PPAE-I-H. PCR was carried out in a 25 μl reaction mixture made from the integument cDNA library (0.5 μl), 25 pmol of each primer, and 1.25 units of Taq polymerase (Life Technologies, Inc.). Based on the integument PPAE cDNA sequence, oligonucleotide primers were designed. The sequences and the names given to the primers are listed in Table I. For 5'-RACE, 0.63 μg of total RNA from hemocytes was reverse-transcribed with Superscript II (Life Technologies, Inc.) using the primer SP1r. After the cDNA synthesis, the cDNA was incubated with terminal deoxynucleotidyl transferase to add a homopolymeric tail of deoxyctydine residues. The cDNA was then diluted 100 times and used directly for further PCR. The first round of PCR was performed using the Abridged Anchor Primer, SP1r, and Taq polymerase for 35 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C. The PCR product was purified using the Abridged Anchor Primer, SP1r, and Taq polymerase for 35 cycles under the following conditions: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C, and extension for 2 min at 72 °C. The first PCR product was used for the secondary “nested” PCR using the Universal Amplification Primer and SP2r. The secondary PCR was performed under the same conditions as above except for annealing at 60 °C for 1 min. The 3'-RACE was performed by employing the same procedure as that for 5'-RACE, except that the oligo(dt)-containing adoptor primer, universal adopter primer, and SP6f were used.

**RT-PCR**—Total RNA (0.63 μg) from hemocytes was used for RT-PCR using the Superscript One-step RT-PCR System (Life Technologies, Inc.). The cDNA synthesis was carried out at 45 °C for 30 min. The subsequent PCR was performed according to the manufacturer’s instructions. Cycling conditions were: 30 s at 94 °C, 30 s at 60 °C, and 2 min at 72 °C. The cycle was repeated 40 times. The oligonucleotide primers for the RT-PCR and PCR are given in Table I. The pairs of primers used were as follows: SP3f and SP1r, SP3f and SP6r, SP4f and SP2r. The PCR products obtained were amplified again under the same conditions as the same pair of primers by PCR before the analyses of the DNA sequences.

**Direct Sequencing of PCR Products**—PCR products were electrophoresed and stained with ethidium bromide. PCR products with the expected size were excised from agarose gel, purified by using Genepure (Nippongene), and subjected to DNA sequencing.

**DNA Sequencing**—DNA sequencing was performed with an auto-
molecular DNA sequencer (model 377, PE Applied Biosystems). Homology Search—All sequence data were analyzed by GENETYX System, version 8.0 (Software Development Co., LTD Tokyo). A homology search was carried out by using SWISS-PROT (Release 36.0) and PIR (Release 57.0).

**Detection of Protein**—Protein was determined by the method of Lowry et al. (34) with bovine serum albumin as a standard.

**RESULTS**

**Purification of PPAE**

Dohke (16) extracted PPAE from the cuticles of silkworms with deionized water and reported purification of the enzyme. We obtained the ammonium sulfate fraction of PPAE (PPAE-dw) by adopting his procedures for the extraction and ammonium sulfate fractionation. We re-extracted the residue of the water extraction with buffer containing 0.5 m NaCl and subjected the extract to ammonium sulfate fractionation between 0.2 and 0.4 saturation to obtain a PPAE fraction (PPAE-salt). We found that PPAE-salt contained much more (16.5 times more) PPAE than PPAE-dw and that the specific activity of PPAE-salt was 19 times higher than that of PPAE-dw. PPAE in PPAE-salt was purified by column chromatography on DEAE-cellulose, hydroxyapatite, and CM-cellulose according to the method of Dohke. In the chromatography, PPAE appeared to be eluted at the essentially same salt and buffer concentrations as those reported by Dohke (data not shown). PPAE in the CM fraction obtained after CM-cellulose column chromatography was further purified by column chromatography on Sepharose-mABA and hydroxyapatite as described under “Experimental Procedures.” The column chromatography on Sepharose-mABA was very effective for removing contaminated proteins (Table II). In the second column chromatography on hydroxyapatite, elution profiles of protein and PPAE activity were symmetrical and superimposable (Fig. 1), indicating that the purity of PPAE eluted from the column was high. About 20 mg of homogeneous PPAE was obtained, and the preparation was named the second HA fraction (Table II).

**Reversed-phase C8 Column Chromatography of PPAE**

Purified PPAE in the second HA fraction was eluted in two peaks in reversed-phase C8 column chromatography (Fig. 2). Proteins with a shorter retention time and with a longer retention time were named PPAE-I and PPAE-II, respectively. PPAE-I and PPAE-II could be separated in reversed-phase chromatography on columns such as a C8 column and ODS column but not by any other fractionation methods examined so far, including polyacrylamide gel electrophoresis at pH 9.2 and 6.5 under nondenaturing conditions, iso-electric polyacrylamide gel electrophoresis (data not shown), and ConA-Sepharose column chromatography.2

Molecular Mass of PPAE

The molecular mass of PPAE under nondenatured conditions was determined to be 45,000 Da in gel permeation chromatography on a Sephacryl S200 column (data not shown). PPAE (second HA fraction and a mixture of PPAE-I and PPAE-II) migrated to the position corresponding to that of 45,000-Da protein in SDS-PAGE under nonreducing conditions. Under reducing conditions, PPAE gave two bands (light chain and heavy chain) in SDS-PAGE (Fig. 3a). The light chain and heavy chain of PPAE were determined to be 22.0 kDa and 38.5 kDa, respectively, from their mobilities. In a separate experiment, both PPAE-I and PPAE-II were shown to give the same electrophoretic pattern, as is seen in Fig. 3a. These results indicate that both PPAE-I and PPAE-II are composed from light and heavy chains. In the present report, we refer to the light chain and the heavy chain of PPAE-I as PPAE-I-L and PPAE-I-H, respectively, and those of PPAE-II as PPAE-II-L and PPAE-II-H. In MALDI mass spectrometry, mass numbers of PPAE-I and PPAE-II were determined to be 48,811 and 48,821, respectively (Fig. 4). After S-pyridylethlylation, the mass numbers of PPAE-I-L, PPAE-II-L, PPAE-I-H, and PPAE-II-H were determined to be 18,122, 18,284, 32,848, and 32,834, respectively. All of the mass numbers observed were bigger than those expected from the deduced amino acid sequences. The calculated mass numbers for the S-pyridylethylated light chains (PPAE-I-L and PPAE-II-L) and the S-pyridylethylated heavy chains (PPAE-I-H and PPAE-II-H) were 17,865.6 and 30,294.1, respectively.

**Iso-electric Point of PPAE**

The iso-electric point of PPAE seemed to be higher than 10, because PPAE could not stay in the gel where a pH gradient from 4 to 10 had been established during electrosfocusing. Furthermore, PPAE could not enter into the separating gel of pH 9.2 in PAGE under nondenaturing conditions. The isoelectric point of PPAE predicted from its deduced amino acid sequence was 7.82 (35). The reason for such a large discrepancy between the observed and the predicted isoelectric points is not known.

**Fluorography of [3H]DFP-labeled PPAE-I and PPAE-II**

PPAE was treated with [3H]DFP before the separation of PPAE-I and PPAE-II by reversed-phase ODS column chromatography. The results in Fig. 3b indicate that PPAE-I-H and PPAE-II-H were labeled with [3H]DFP but PPAE-I-L and PPAE-II-L were not. Thus, both PPAE-I-H and PPAE-II-H were indicated to be catalytic polypeptides of serine proteases.

**Detection of Carbohydrates of PPAE-I and PPAE-II by SDS-PAGE and Lectin Blotting**

None of the lectins used in the present study bound to PPAE-I-L and PPAE-II-L under the experimental conditions (Fig. 5). PPAE-I-H and PPAE-II-H were blotted by concanavalin A, lentil seed agglutinin A, and Dolichos biflorus agglutinin but...
Prophenoloxidase-activating Enzyme of the Silkworm

Some Enzymatic Properties of PPAE

Because we could not separate PPAE-I and PPAE-II by any means other than reversed-phase chromatography, we used a mixture of the isoforms to examine the enzymatic properties.

Thermostability—PPAE was stable at 25 °C, but above that temperature, inactivation occurred. At 50 °C, PPAE lost its activity completely in 5 min (Fig. 6a). pH stability at 0 °C for 24 h: PPAE was shown to be stable in a pH range of 6.0 to 8.5. Below pH 6.0, inactivation occurred, and PPAE lost its activity completely during incubation at pH 3.5. In alkaline pH, PPAE was relatively stable, and 80% of the activity remained even at pH 10.5 (Fig. 6b).

Effect of the Concentration of Salt on Esterase Activity—The esterase activity of PPAE was greatly affected by the salt concentration. The ability of PPAE to hydrolyze BAEE at 4 mM KCl was less than 10% of that at 500 mM KCl (Fig. 7a). KCl was shown to have an effect on $K_m$ and $V_{max}$ of PPAE when its activity was assayed by using BAEE as a substrate. At low concentrations (4–40 mM) of KCl, $K_m$ for BAEE was about 1 mM, and at high concentrations, $K_m$ decreased to 0.35 mM at 250 mM KCl. The value of $V_{max}$ obtained at 250 mM KCl was about 4.5 times higher than that obtained at 4 mM KCl (Fig. 7b).

Substrate Specificity—Substrate specificity of PPAE was examined by using various fluorogenic substrates, NH-Mecs (Table III). NH-Mecs were required to have Arg at the P1 site to be hydrolyzed efficiently by PPAE. However, Arg at the P1 site was not sufficient for the substrates to be hydrolyzed. The amino acids at P2 and P3 sites also affected the ability of the substrates to be hydrolyzed. Substrate specificity of BAEEase (15), which has been shown to be activated in the silkworm plasma from pro-BAEEase by microbial cell wall components, was different from that of PPAE.

Molecular Cloning of the Integument PPAE cDNA

The six partially overlapping clones were screened from the Lambda ZAPII integument cDNA library. These clones were subcloned into pBluescript SK and subjected to DNA sequence analyses. Of these six clones, the one with the longest insert contained 1447 bp of PPAE cDNA (Fig. 8). The insert included 43 bp of 5'-untranslated region, 1323 bp of an open reading frame, a TGA termination codon, 60 bp of 3'-untranslated region, and 18 bp of a poly(A) tail. A polyadenylation signal, AATAAA, was found at 28 bases upstream of the poly(A) tail. The open reading frame, nt 43–1366, encoded a polypeptide consisting of 441 amino acid residues and terminated in front of the TGA termination codon. The N-terminal sequences of PPAE-I and PPAE-II were pyroglutamy1-SCRTPNGLN.

Some Enzymatic Properties of PPAE

Because we could not separate PPAE-I and PPAE-II by any means other than reversed-phase chromatography, we used a mixture of the isoforms to examine the enzymatic properties.
bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.3 kDa).

of the observed Mass/H were examined for each of PPAE-I and PPAE-II, and the mean values of the mass spectrometry are described under “Experimental Procedures.”

Digestion of purified PPAE on the storage. Details of the mass spectrometry gave very faint bands under the 45-kDa PPAE band. Substances giving mass numbers between MH1 and MH2 seemed to be proteins stained faintly in the SDS-PAGE. We have observed self-agglutination from the integument, hemocytes and salivary glands of silkworms on day 3 of the fifth instar by Northern blot analysis using a PPAE cDNA probe. The most intense hybridization signal was observed with the RNA preparation from the integument, and the intensity decreased with hemocytes and with salivary glands in this order. PPAE mRNA was not detected in RNA preparations from the fat body, mid gut, and silk gland (Fig. 9).

Activation Mechanism of pro-PPAE

From the lysyl endopeptidase digest of the S-pyridylethylated PPAE-I-L, we could isolate a peptide containing the amino acid sequence NH2-CCGRDIAVGDK-COOH. This sequence is followed by an N-terminal sequence of PPAE-I-H in the deduced amino acid sequence. We could also isolate a peptide containing the C-terminal sequence of the deduced amino acid sequence. We could also isolate a peptide containing the C-terminal sequence of the deduced amino acid sequence.

Tissue-specific Expression of PPAE mRNA

Transcripts of 1.5 kilobases were detected in RNA preparations from the integument, hemocytes and salivary glands of silkworms on day 3 of the fifth instar by Northern blot analysis using a PPAE cDNA probe. The most intense hybridization signal was observed with the RNA preparation from the integument, and the intensity decreased with hemocytes and with salivary glands in this order. PPAE mRNA was not detected in RNA preparations from the fat body, mid gut, and silk gland (Fig. 9).

Southern Blot Analysis

The number of PPAE genes in the genome was estimated by Southern blot analysis (Fig. 10). The probe fragment was a SalI/HindIII digest of PPAE cDNA, corresponding to 439–1070 nt, and did not have any site for the restriction enzymes used in the present analysis. We found one to three hybridization bands with different mobilities in the digests obtained by treating the genomic DNA with eight restriction enzymes. More than one hybridization band was observed after EcoRI, HindIII, and PstI digestion of the genomic DNA. However, only one
hybridization band was detected in the other digests. The three hybridization bands from EcoRI and PstI digests suggest the presence of more than two introns and more than one intron, respectively, in the region of the genome containing the sequence of the probe fragment.

Hemocyte PPAE cDNA Sequence

The hemocyte PPAE cDNA sequence was determined by RT-PCR and 5′- and 3′-RACE analyses. The hemocyte cDNA was shown to have a sequence different from that of integument PPAE cDNA at six bases, as stated in the legend to Fig. 8. At the protein level, however, the difference results in only a substitution from Gly202 to Ala202.

Detection of a Polypeptide Cross-reactive to Anti-PPAE/IgG

A polypeptide was detected after SDS-PAGE and immunoblotting of SDS/urea extract of cuticles of silkworm larvae on day 6 of the fifth instar. Under nonreducing conditions, the mobility of the blotted polypeptide was the same as that of purified PPAE. Under reducing conditions, however, the polypeptide in the SDS/urea extract cross-reactive to anti-PPAE/IgG migrated to a position corresponding to the mobility of a 53-kDa protein (Fig. 11). The 53-kDa protein seems to be a zymogen form of PPAE. In plasma, a polypeptide cross-reactive to anti-PPAE/IgG migrated to a position corresponding to the mobility of the blotted polypeptide in the SDS/urea extract cross-reactive to anti-PPAE/IgG and with the same mobility in SDS-PAGE as that of cuticular PPAE. A polypeptide cross-reactive to the antibody was not detected in extracts prepared from other developmental stages.

DISCUSSION

Purification of PPAE from larval cuticles of silkworm, B. mori, and some properties of the enzyme were reported by Dohke about 26 years ago (16). Since then, no further characterization of the enzyme has been reported. Since Dohke's work, PPAE has been demonstrated to be a member of a protease cascade (pro-PO cascade), which is now recognized as one of the major defense mechanisms of insects and is speculated to interplay with other defense mechanisms. To understand the mechanism of the interplay, thorough investigation of the properties of each component of the pro-PO cascade is necessary. In the present study, we developed an improved purification procedure to obtain homogeneous PPAE in high yield from larval cuticles of the silkworm, B. mori, and we examined the properties of the enzyme by biochemical, immunochemical, and molecular biological techniques. The introduction of an extraction method using a buffer with a high salt concentration (0.5 M NaCl) and column chromatography on Sepharose-PRO were extremely effective for obtaining PPAE in high yield and high purity in the present purification from cuticles of the silkworm, B. mori. One of the present
authors has previously reported that pro-PPAE in the extract of silkworm cuticles is activated to PPAE if CaCl2 is present in the extract (10). Because we employed a buffer containing CaCl2 for extracting PPAE, pro-PPAE in cuticles is likely to be activated during the extraction. We could obtain about 20 mg of PPAE from the cuticles of 13,000 larvae on day 5 or 6 of the fifth instar. In SDS-PAGE, the purified PPAE gave a single band at 45.0 kDa under nonreducing conditions and two bands (heavy chain and light chain) at 38.5 and 22.0 kDa under reducing conditions.

The mass number of PPAE-I and PPAE-II observed in MALDI spectrometry (Fig. 4), and those of PPAE, its light and heavy chains observed in SDS-PAGE (Fig. 3a) were not consistent. Considering the accuracy of MALDI mass spectrometry, PPAE, its light chain, and its heavy chains seem to indicate that they behave anomalously in SDS-PAGE. The reason why the sum of observed mass number of light chain and heavy chains was larger than the one observed for PPAE under nonreducing conditions in SDS-PAGE is not clear. However, a similar phenomenon has been observed on the proclotting enzyme of horseshoe crab (36).

Our results are not consistent with those in Doheke's report (16) in which PPAE was estimated to be a 33-kDa protein in SDS-PAGE under reducing conditions and to be composed of a single polypeptide. As Doheke extracted cuticles with deionized and distilled water, we examined whether our AS fraction-dw (prepared from the water extract of cuticles) contained the protein cross-reactive to monospecific anti-PPAE/IgG prepared in the present study. By SDS-PAGE and immunoblotting of AS fraction-dw, we could detect a polypeptide of which the mobility was the same as that of the PPAE purified in the present study (data not shown). This result indicates that PPAE extracted from cuticles with deionized and distilled water is the same molecule as PPAE extracted under a high salt concentration. The reason for the discrepancies in molecular mass and subunit structure between Doheke's PPAE and ours remains to be clarified. Both PPAE preparations by Doheke and by us in the present study could activate silkworm pro-PO without the participation of any other factor (16), contrasting the silkworm PPAE with coleopteran PPAE (20), which has been reported to require another proteinaceous factor to convert the respective pro-PO into the active PO. Chosa et al. (19) have also observed that *Drosophila* PPAE could activate pro-PO by itself by using homogeneous preparations of pro-PO and PPAE.

We examined the following enzymatic properties of purified PPAE: substrate specificity (Table III), effects of KCl on *Km* and *V* max in hydrolyzing BAEE, and pH stability and thermostability. In the principal points, the results confirmed and extended what Doheke reported 26 years ago. Among NH-Mecs used as substrates, those with Arg at the P1 site were hydrolyzed. This result is consistent with Doheke's observation that BAEE and tosyl-1-arginine methyl ester were hydrolyzed but tosyl-1-lysine methyl ester was not. PPAEs of the fruit fly (*D. melanogaster*) and beetle (*Holotrichia diomphalia*) have been examined for their substrate specificity using NH-Mecs (19, 20). PPAEs of the silkworm, fruit fly, and beetle seem to have the common feature of exhibiting a preference for the substrate with Arg at the P1 site. The fruit fly PPAE and silkworm PPAE hydrolyze Boc-Val-Pro-Arg-Mec best among the substrates so far examined, but Boc-Phe-Ser-Arg-Mec has been reported to be the better substrate for the beetle PPAE than Boc-Val-Pro-Arg-Mec. We previously reported that another serine protease of the pro-PO cascade, BAEE, also hydrolyzes NH-Mecs with Arg at the P1 site (15). However, the preferences of PPAE and BAEE for NH-Mecs with Arg at P1 site as substrates were different (Table III). We recently showed that PPAE hydrolyzes only the peptide bond between R and F in **N**<sup>49</sup>R<sup>50</sup>F<sup>51</sup>G<sup>52</sup> sequences of pro-PO polypeptides I and II (37).

This observation seems to indicate that PPAE is an enzyme demanding strict requirements to its proteinaceous substrates. *Km* and *V* max of PPAE decreased and increased, respectively, in response to the increase in KCl concentration in the reaction mixture when BAEE was used as a substrate. *Km* was 1.2 mM and 0.35 mM at 4 mM and 500 mM KCl, respectively. *V* max at 4 mM KCl was about one-fourth of that at 500 mM KCl. Contrary to the observation with BAEE, the ability of PPAE to activate pro-PO was not enhanced under high salt concentrations (16). We have observed that the molecular size of pro-PO does not change after incubation with PPAE in the presence of 0.5 mM KCl, indicating that the peptide bond in **N**<sup>49</sup>R<sup>50</sup>F<sup>51</sup>G<sup>52</sup> sequences of pro-PO is not hydrolyzed under these conditions. It is possible that conformational change of pro-PO due to high salt concentration caused the steric hindrance for PPAE to form an enzyme-substrate complex.

Although the purified PPAE was judged to be homogeneous by SDS-PAGE (Fig. 3a) and elution profiles in the column chromatography on hydroxyapatite (Fig. 1) and Sephacryl S-200, PPAE was resolved into two fractions in HPLC on a C<sub>8</sub> column. PPAE eluted at shorter and longer retention times was named PPAE-I and PPAE-II, respectively. Both PPAEs are composed of a light chain and heavy chain, which are linked by disulfide linkage(s). The difference between the light chains and between the heavy chains could not be detected in such properties as the mobility in SDS-PAGE, N-terminal amino

| Substrate<sup>a</sup> | Relative hydrolyzing activity<sup>b</sup> |
|----------------------|--------------------------------------|
| Bz-Arg-Mec           | 0.0                                  |
| Z-Arg-Mec            | 0.0                                  |
| Boc-Gln-Arg-Mec      | 200                                  |
| Boc-Ala-Gly-Pro-Arg-Mec | 0.7              |
| Boc-Asp(Obz)-Pro-Arg-Mec | 9.9                        |
| Boc-Glu(Obz)-Gly-Mec | 13.8                                |
| Boc-Ile-Glu-Gly-Mec  | 5.7                                  |
| Boc-Leu-Ala-Gly-Mec  | 1.0                                  |
| Boc-Leu-Thr-Arg-Mec  | 2.9                                  |
| Boc-Leu-Ser-Thr-Arg-Mec | 7.5                   |
| Pro-Phe-Arg-Mec      | 5.6                                  |
| Z-Phe-Arg-Mec        | 34.7                                 |
| Boc-Glu-Lys-Lys-Mec  | 0.0                                  |
| Suc-Ala-Pro-Ala-Mec  | 0.2                                  |
| Boc-Phe-Ser-Arg-Mec  | 72.9                                 |

<sup>a</sup> Substrates other than those indicated here were examined. However, none of them were hydrolyzed appreciably by PPAE under the present experimental conditions. The substrates that were not hydrolyzed were as follows: Arg-Mec, Boc-Arg-Val-Arg-Mec, Boc-Gln-Arg-Arg-Mec, Boc-Val-Leu-Lys-Mec, Gly-Ala-Ala-Phe-Mec, Gly-Gly-Arg-Mec, Gly-Pro-Mec, Leu-Mec, Lys-Ala-Mec, Phe-Mec, Pyr-Arg-Thr-Lys-Arg-Mec, Suc(OMe)-Ala-Ala-Pro-Mec, Suc-Ala-Ala-Pro-Phe-Mec, Suc-Arg-Pro-Phe-his-Leu-Leu-Val-Tyr-Mec, Suc-Gly-Pro-Leu-Gly-Pro-Mec, Suc-Gly-Pro-Mec, Suc-Gly-Pro-Val-Tyr-Mec, Suc-Ile-Ala-Mec, Suc-Leu-Leu-Val-Tyr-Mec, Z-Vals-Met-Mec.

<sup>b</sup> Data for BAEEase were taken from Katsumi et al. (15).
acid sequence, reactivities to lectins, and tritiated DFP. N-terminal amino acids of light chains of PPAE-I and PPAE-II were determined to be pyroglutamic acid. Furthermore, practically the same mass numbers, 48,811 and 48,821 for PPAE-I and PPAE-II, respectively, were observed in MALDI mass spectrometry (Fig. 4). The structural basis for causing the difference in the retention times and mass numbers of PPAEs I and II will be discussed later in this section with the results on cDNA sequencing. Because we could not find any means to

FIG. 8. Nucleotide sequence of the integument PPAE cDNA and the deduced amino acid sequence. The nucleotide sequence (upper) and the deduced amino acid sequence (lower) are shown. The amino acid sequence is numbered from the amino terminus of the mature protein. The underlined amino acid residues were confirmed by protein sequencing. The cleavage sites accompanied by maturation of the nascent pro-PPAE polypeptide and activation of pro-PPAE are shown by an open arrow and a closed arrow, respectively. The terminal codon is marked with an asterisk. The putative polyadenylation signal is indicated by a double underline. The predicted attachment sites for N-linked carbohydrate chains are indicated by open triangles. The active triad of the serine protease domain is indicated by closed circles. The nucleotide sequence of hemocyte PPAE cDNA was determined by RT-PCR 5′-RACE and 3′-RACE as described under “Experimental Procedures.” The cDNA was found to be longer by the sequence 5′-ATTTCGCTCAGAACAGCAACAAAGTTCCGAACCT-3′ than integument cDNA in the noncoding 5′ sequence. The sequence was found to be attached to the 5′ end of integument cDNA. The sequence in the open reading frame of the hemocyte PPAE cDNA was different from that of integument PPAE cDNA at five nucleotide residues. The nucleotide residues A222, C250, T344, G711, A712, and G928 of integument PPAE cDNA are replaced by G, T, C, C, G, and A, respectively, in hemocyte PPAE cDNA. At the amino acid level, the deduced hemocyte pro-PPAE amino acid sequence was the same as the integument pro-PPAE sequence except that Gly202 of cuticle pro-PPAE was replaced by Ala.
separate the isozymes other than reversed-phase HPLC and because PPAE lost its activity during the HPLC, we could not examine whether there are functional differences between PPAE-I and PPAE-II.

The carbohydrate moieties of PPAE-I and PPAE-II were indicated to attach to Asn residues (Asn\(^{218}\) and Asn\(^{313}\)) of potential N-linked glycosylation sites found in the deduced amino acid sequence of PPAE. Considering the indications, the results of SDS-PAGE and lectin blotting could be explained as follows: (a) Both PPAE-I-H and PPAE-II-H have α-1,6-N-acetylgalactosamine and (b) One or both of the N-linked carbohydrate chains are complex-type and have α-d-fucose attached by (α-1,6) linkage to N-linked α-N-acetylgalactosaminine. The mass numbers of S-pyridylethylated PPAE-I-H and PPAE-II-H observed in MALDI mass spectrometry were larger by 2,554 and 2,540, respectively, than the mass number calculated from the deduced amino sequence of the heavy chain, of which cysteine residues were assumed to be S-pyridylethylated. The difference between the observed and calculated mass numbers could account for two N-linked carbohydrate chains and is thus consistent with the fact that the residues of Asn\(^{218}\) and Asn\(^{313}\) seemed to be linked to carbohydrate chains.

The longest cDNA of PPAE screened from the cDNA library of body wall epidermis of a larva on day 3 of the fifth instar, was 1,447 bp. It encoded a single open reading frame consisting of a 21-amino acid prepropeptide followed by a 420-amino acid mature protein. We obtained results indicating that the mature protein. The longest cDNA of PPAE screened from the cDNA library of body wall epidermis of a larva on day 3 of the fifth instar, was 1,447 bp. It encoded a single open reading frame consisting of a 21-amino acid prepropeptide followed by a 420-amino acid mature protein. We obtained results indicating that the mature protein contains a typical catalytic domain of trypsin-type serine protease, with active site triads, His\(^{187}\), Asp\(^{264}\), and Ser\(^{370}\). Labeling of the heavy chain with tritiated DFP (Fig. 3b) supports the notion that both triads in PPAE-I and PPAE-II function as an active site.

![Fig. 11](image1) Detection of polypeptides cross-reactive to anti-PPAE/IgG by SDS-PAGE and immunoblotting. a, SDS-PAGE pattern stained by CBB. SDS-PAGE was done under reducing conditions. Samples applied to the lanes: lane 1, SDS extract of eggs (7 μg); lane 2, plasma of silkworm hemolymph (10 μl); lane 3, SDS/urea extract of cuticles (10 μl). b, immunoblotting. SDS-PAGE was done under nonreducing conditions. Samples: lanes 1, 2, and 3, same as in a; lane 4, 0.12 μg of PPAE (second HA fraction); lane M, marker proteins. c, immunoblotting. SDS-PAGE was done under reducing conditions. Samples: lanes 2 and 3, same as in a; lane 4, 0.60 μg of PPAE (second HA fraction). Under reducing conditions, SDS extract of eggs did not give any polypeptide cross-reactive to anti-PPAE/IgG under the present experimental conditions. We have noticed that PPAE and the cross-reactive polypeptide in SDS/urea extract of cuticles were much more reactive to anti-PPAE/IgG under nonreducing conditions than under reducing conditions. SDS extract of eggs was run in 10% separating gel, and other samples were run in 12% separating gel. The mass number of marker proteins in kDa at the left of b is for calibration of lane 1. Lane M is for calibration of lanes 2, 3, and 4 in b and c.

The hemocyte PPAE cDNA sequence was determined by RT-PCR and 5'- and 3'-RACE analyses. Although the cDNA was longer by 34 bp than the integument PPAE cDNA at the 5' upstream region, sequences of an open reading frame and 3'-untranslated region were shown to be the same as the integument PPAE cDNA, except that substitutions of a base at six nucleotide residues were observed in the open reading frame. Among six substitutions, only the substitution of guanine 710 to cytosine 710 caused the substitution of an amino acid from Gly\(^{202}\) to Ala\(^{202}\). Considering the technique used for the determination of the hemocyte PPAE cDNA sequence, we could not rule out the possibility that the observed sequence includes artifacts. Therefore, it is not clear whether the gene coding for the PPAE variant with Ala\(^{202}\) really exists in the silkworm genome (Fig. 10), PPAE variants, even if they exist, must be allelic variants. It is possible to explain the presence of PPAE-I and PPAE-II as the result of the expression of allelic genes that code for the PPAEs with Ala\(^{202}\) and Gly\(^{202}\).

In Northern blot analyses, PPAE transcripts were detected in total RNA preparations from the epidermis, hemocytes, and salivary glands but not in those from the fat body and mid gut (Fig. 9). Because PPAE is synthesized as prepro-PPAE, it is likely that the molecule is secreted from the cell where it is synthesized. It has not been elucidated, however, whether prepro-PPAE synthesized in the epidermis is secreted in both directions, to the cuticle and to hemocoel, or only to the cuticle. Furthermore, the type of hemocyte engaging in the synthesis of the prepro-PPAE remains to be studied. The implication of the presence of PPAE transcripts in the salivary gland is not clear at present. By Western blotting, we examined whether the same molecule in terms of mobility in SDS-PAGE is present in both the cuticle and plasma (Fig. 11). In the extract of cuticle with SDS and urea, only protein with mobility corresponding to 53 kDa under reducing conditions and 45 kDa under nonreducing conditions was shown to be cross-reactive to anti-PPAE/IgG. The mass number, 45 kDa, was also observed for the purified PPAE in SDS-PAGE under nonreducing conditions. The above results seem to show that the present anti-PPAE/IgG is mono-specific to PPAE and the zymogen form of PPAE.
We observed that the purified PPAE zymogen is a protein with mobilities corresponding to 45 and 53 kDa under nonreducing conditions and reducing conditions, respectively. When plasma was examined for the presence of pro-PPAE by Western blot analysis, no cross-reactive band was detectable (Fig. 11, b and c).

Previously, we could detect PPAE activity in plasma treated with a microbial cell wall component, and we also obtained results suggesting that PPAE in plasma cleaved pro-PO subunits at the same peptide bonds as cuticular PPAE (38). Furthermore, we detected the same PPAE transcripts in hemocytes as those in the body wall epidermis in the present study. Therefore, the reason why we could not detect PPAE (or pro-PPAE) in plasma seems to be the low concentration of the zymogen, which was under the limit of detection by the present method of Western blot analysis.

We searched for proteins with similarity to the PPAE sequence. The proteins with similarity (the degree of their similarity is indicated in parentheses) were as follows: Drosophila serine protease zymogens, easter (35.4%) (39) and snake (25.3%) (40), and horseshoe crab (Tachypleus tridentatus) serine protease zymogens, proclotting enzyme (27.4%), and Factor B (26.8%). Thesezymogens are known as the components of invertebrate protease cascades such as the dorso-ventral pathway in the perivitelline space of the developing Drosophila embryo and the blood coagulation cascade in the hemolymph of the horseshoe crab. The above proteins were demonstrated, or speculated, to have a so-called “clip-like domain” or “disulfide knot domain” (41, 43) in which six cysteine residues form three disulfide linkages. As is shown in Fig. 12, the light chain of PPAE seemed to contain two consecutive clip-like domains. To our knowledge, a protease zymogen with two clip-like domains has not been reported before in the literature, although pro-clotting enzyme (41) and Factor B (42). All of the residues have been indicated to be O-glycosylated. MALDI mass spectrometry of S-pyridylethylated light chains of PPAE-I and PPAE-II showed that their mass numbers are larger by 247 and 419, respectively, than those calculated from the deduced amino acid sequence in which cysteine residues were assumed to be S-pyridylethylated. This difference in mass numbers seems significant if we consider the accuracy of the present MALDI mass spectrometry. Thus, the present results do not exclude the possibility of the presence of O-glycosylated threonine residue(s) in the PPAE light chain. We noticed that the PPAE light chain showed anomalous behavior: lower mobility in SDS-PAGE than expected and less stainability with CBB. The same anomalous properties have been observed for the light chain of proclotting enzyme (47).

The pro-PO cascade (pro-PO activating system) of the silk-worm hemolymph has at least one reaction step where Ca$^{2+}$ ions are required (48). The γ-carboxyglutamic acid domain is known to be present in some proteases and to bind Ca$^{2+}$ ions for the activation of proteases in the mammalian blood coagulation system (49). Therefore, one can expect to find a γ-carboxyglutamic acid domain in one of the proteases in the pro-PO cascade. However, such a domain was not detected in the deduced amino acid sequence of pro-PPAE. The absence is consistent with our previous observation that PPAE does not require Ca$^{2+}$ ions to activate pro-PO. In the cascade of the dorso-ventral pathway of Drosophila, a protease, snake, which converts pro-easter to easter, was shown to have a γ-carboxyglutamic acid domain (40). Future studies may show that protease(s) upstream of PPAE in the pro-PO cascade have such a domain.

Catalytic subunits of serine proteases have conserved sequences especially around amino acids participating in the catalytic action and the formation of disulfide linkages (50). The sequence of the heavy chain of PPAE was found to be similar to the catalytic subunits of the following proteases (the degree of similarity in parentheses): prothrombin (30.8%) (51), factor IX (32.3%) (52), factor XI (33.7%) (53), human plasma kallikrein (35.2%) (54), and Limulus proclotting enzyme (29.7%) (41). From the alignment of these sequences, we learned that disulfide linkages are likely to be formed between cysteine residues of PPAE. In fact, PPAE contains three cysteine residues, Cys$^{355}$, Cys$^{366}$, and Cys$^{385}$. Furthermore, in the light chain of PPAE, one of cysteine residues, except those being engaged in the formation of two clip-like domains, seemed to be linked to Cys$^{304}$ of the heavy chain by a disulfide linkage.

Before the present study, insect proteases whose functions are known and that have a clip-like domain have been reported only from the protease cascade in the dorso-ventral pathway, which operates in perivitelline space at the early embryonic
stage of *D. melanogaster*. Easter and snake are such proteases. Snake activates easter, which, in turn, converts prospätzle to spätzle. As was mentioned in the Introduction, the protease cascade with easter or a protease with a substrate specificity similar to that of easter have been shown to be involved in the extracellular signaling pathway for the ventralization of blastodermal cells and for the induction of antifungal peptide synthesis of the adult *Drosophila* fat body. To date, the pro-PO cascade is the only protease cascade that could be activated by one of fungal cell wall components, β-1,3-glucan, and is present in both larval and adult stages of insects. In the present study, pro-PPAE of the pro-PO cascade was shown to be homologous to easter. Furthermore, preliminary immunohistological study showed that silkworm eggs at 2 h after being laid have a polypeptide cross-reactive to the mono-specific anti-PPAE/IgG and that the mobility of the polypeptide is about the same as that of purified PPAE (Fig. 11). Our present observations that have been reported so far about the doro-ventral pathway, the extracellular signaling pathway for activation of the drosomycin gene, and the indication of the presence of pro-PO cascade in silkworm eggs (55) imply that the pro-PO cascade may operate not only for the synthesis of melanin from phenolic compounds but also in developmental events in the early embryonic stage and in the signaling pathway for activation of immune protein gene(s).

After submission and during the review of the present paper, two papers dealing with prophenoloxidase-activating enzyme appeared (56, 57). The enzymes from lepidopteran (*Manduca sexta*) and coleopteran (*H. diomphalia*) were reported to be serine proteases as ours but require another proteinaceous factor to make them competent to activate pro-PO and have one clip-like domain. From the reported sequences, the similarity of the silkworm PPAE to the *Manduca* PPAE and Holotrichia PPAE is calculated to be 34.7 and 35.0%, respectively. The *Holotrichia* PPAE has been claimed to hydrolyze peptide bond at two sites in each of pro-PO subunits when it works with auxiliary proteinaceous component (20). As described in the present paper and as reported before (6, 10, 16, 21, 37, 58–60), the silkworm PPAE is different in these properties. Thus, there seem to be two kinds of serine-type proteinase among insects that are directly involved in the activation of pro-PO. It remains to be seen whether the two kinds of PPAE are present in each of insect species and, if so, what the physiological significance of their presence is. Apparently, more thorough investigations of the pro-PO cascade components are needed to answer the questions surrounding the pro-PO cascade.

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