Cuticular Pro-phenoloxidase of the Silkworm, *Bombyx mori*

PURIFICATION AND DEMONSTRATION OF ITS TRANSPORT FROM HEMOLYMPH*

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Pro-phenoloxidase (proPO) in insects is implicated in the defense against microbes and wounding. The presence of proPO in the cuticle was suggested more than 30 years ago, but it has not been purified. The extract of cuticles of the silkworm, *Bombyx mori*, was shown to contain two proPO isoforms (F-type and S-type proPOs, which have slightly different mobilities in polyacrylamide gel electrophoresis under nondenaturing conditions). The two isoforms were purified to homogeneity. From hemolymph of the same insect, two types of proPO with the same electrophoretic mobilities as those of cuticular isoforms were separated and were shown to be different at five amino acid residues in one of their subunits. The isoforms in the hemolymph and cuticle were activated by a specific activating enzyme. The resulting active phenoloxidases exhibited almost the same substrate specificities and specific activities toward o-diphenols. The substrate specificities and the susceptibilities to inhibitors, including carbon monoxide, indicated that the purified proPO isoforms were not zymogens of laccase-type phenoloxidase. The proPO in hemolymph was shown to be transported to the cuticle. This demonstration was corroborated by the failure to detect proPO transcripts by Northern analysis of total RNA from epidermal cells. In reversed-phase column chromatography, cuticular and hemolymph proPOs gave distinct elution profiles, indicating that some yet to be identified modification occurs in hemolymph proPO and results in the formation of cuticular proPO. There was little transportation of cuticular proPO to the cuticle when it was injected into the hemocoel. The nature of the modification is described in the accompanying paper (Asano, T., and Ashida, M. (2001) *J. Biol. Chem.* 276, 11113–11125).

The outer surface of arthropods, including the insect, is covered by a protective armor, the cuticle. The cuticle is a nonliving matrix of carbohydrates and proteins secreted by the underlying monolayer of epithelial cells (1). Because of its strategic localization between the environment and internal organs, the cuticle has been considered to be the first line of defense that is indispensable to safeguard the homeostasis of the body. At the same time, it has often been evaluated only as a nonliving physical barrier. This view, however, is changing dramatically because of the recent demonstration of active participation of the cuticle in the defense reaction against microbial invasion and in the storage of proteins (2, 3).

The cuticle has been shown to have a certain mechanism to sense the presence of bacteria and fungi in an injured part and to relay signals to the underlying epithelial cells to direct the synthesis of a bacteriocidal peptide and its secretion to the cuticular matrix (3). However, it is still not clear what kind of mechanism is involved in the signaling in the cuticle. Interestingly, the existence of a pro-phenoloxidase (proPO) cascade (proPO-activating system) in the cuticle of the silkworm, *Bombyx mori*, has been reported (4). In the 1980s, this cascade has been shown to be present also in the hemolymph of this insect and to be composed of recognition systems with specific affinity to microbial cell wall components, serine protease zymogens and proPO (5). The cascade is triggered by very minute amounts of cell wall components such as peptidoglycan, β-1,3-glucan and possibly lipopolysaccharide. It is considered to be one of the recognition systems for bacteria and fungi (5). Once the proPO cascade is triggered, one of the results is activation of proPO. Active phenoloxidase (PO) is a key enzyme for oxidation of phenolic substances such as tyrosine, DOPA, and dopamine to melanin (6). During the oxidation, quinones are formed in the melanized cell layers surrounding the encapsulated microbes, which are too big to be phagocytosed (7). These quinones are thought to be cytotoxic and facilitate the killing of encapsulated microbes. It is possible that the cascade in the cuticle also plays a similar role (4).

Epithelial cells underneath the cuticle secrete proteins. The secretion is directed apically to the cuticular matrix or basolaterally to the hemolymph or bidirectionally to the cuticle and hemolymph (8). In addition to this secretion, the epithelial cells have been shown to be capable of transcytosis of proteins, a process in which the epithelial cells take up proteins from the hemolymph and secrete them apically to the cuticle or vice versa. Thus, the cuticle has been shown to function as a reservoir of proteins (2).

What are the underlying mechanisms by which these physiological functions of the cuticle and the epithelial cells are exhibited? How are the components playing roles in the cuticular functions organized in the cuticular matrix? Almost noth-

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1 The abbreviations used are: proPO, pro-phenoloxidase; PO, phenoloxidase; proPO-CS, S-type cuticular proPO; proPO-CF, F-type cuticular proPO; proPO-HS, S-type hemolymph proPO; proPO-HF, F-type hemolymph proPO; PPAE, pro-phenoloxidase-activating enzyme; PAGE, polyacrylamide gel electrophoresis; RP, reversed-phase; HPLC, high performance liquid chromatography; ODS, octadecyl; AcCN, acetonitrile; Lys-C, lysylendopeptidase; CBB, Coomassie Brilliant Blue R-250; DOPA, L-3,4-dihydroxyphenylalanine; DA, dopamine; NADA, N-acetyldopamine; PCR, polymerase chain reaction; kbp, kilobase pair(s).
Pro-phenoloxidase of the Insect Cuticle

Pro-phenoloxidase (proPO) is known to be involved in the melanization process and the immune response in insects. The mechanism of transport of proPO to the cuticle and its activation by proPO-activating enzyme (PPAE) is of particular interest. Here, we report the purification and characterization of isoforms of cuticular proPO and hemolymph proPOs in silkworms, and the properties of the active forms. We also present evidence of the transport of hemolymph proPO to the cuticle and of modifications of its active form.

**EXPERIMENTAL PROCEDURES**

**Silkworm (B. mori)—**Silkworm larvae (Kinsyu × Showa) were reared on the artificial diet Silkmate 2M (Kyodo Shiryo, Tokyo) at 25 °C under a 12-h photoperiod and 70% RH.

**ProPO-activating Enzyme (PPAE)—**Crude PPAE preparation, referred to as PPAE (AS-salt) and purified PPAE preparation were obtained from larval cuticles of silkworms as previously reported (14).

**Assay of PO Activity and Determination of the Amount of ProPO—**

The activity of PO was assayed by the spectrophotometric method that had originally been reported by Pye (15) and has subsequently been used in our laboratory with modifications (16). Briefly, PO was incubated at 30 °C for 5 min in 1.25 ml of a reaction mixture containing 80 mM potassium phosphate buffer, pH 6.0, 8 mM 4-methylcatechol, and 8 mM 4-hydroxyproline ethylester. The increase in absorbance at 520 nm was measured with a spectrophotometer (Shimadzu, model UV-240). One unit of enzyme was defined as the amount causing an increase in absorbance of 0.01 under the above conditions. PO was assayed by this method throughout the present study, unless otherwise specified.

The amount of proPO was quantified by assaying the PO activity after the conversion of proPO to PO by PPAE (AS-salt) at pH 7.5 as described previously (16). One unit of proPO was defined as the amount of the protein that had 1 unit of PO activity upon activation.

**Extraction of Cuticular ProPO Isoforms—**Cuticular proPOs were extracted as described previously (4). Briefly, each cuticle of fifth instar larvae reared to day 5–6 was subjected to extraction of proteins on ice for 1 h by using 1 ml of 50 mM acetic acid, pH 5.2, containing 10 mM EDTA and 33.3 μM p-amidinophenyl-methanesulfonfluoride (Sigma). The extract was centrifuged at 15,000 × g for 10 min at 4 °C to remove the flocculent materials.

**Purification of Cuticular ProPO Isoforms—**

Purification of cuticular proPO, the properties of the proenzyme and the enzymatic properties of its active form must be determined. Furthermore, a clear demonstration of transport of hemolymph proPO to the cuticle and a comparison of the properties of cuticular and hemolymph proPOs should provide clues to understand the mechanism by which hemolymph proteins are transported to the cuticle.

The principal aim of the present paper and the accompanying paper (9) is to get keys for solving the mechanism of transcellular protein transport in insects by using proPO as a probe. Our knowledge on molecular properties of proPOs and their active forms has not reached to the level compatible with the purpose. Neither homogeneous cuticular proPO nor its active enzyme (PO) has been characterized (10), since the original suggestion of the presence of proPO in insect cuticle by Lai-Fook (11). With regard to the hemolymph proPO of lepidopteran insects, the presence of two isoforms has been indicated (12, 13), but they have yet to be isolated and characterized.

Here, we report the purification and characterization of isoforms of cuticular and hemolymph proPOs and some enzymatic properties of the active forms. We also present evidence of transport of hemolymph proPO to the cuticle and of modification occurring in the cuticular proenzymes. On the other hand, we found that cuticular proPO was not transported when it was injected into the hemocoel, indicating that the mechanism for recognition of subtle differences between cuticular and hemolymph proPOs operates in the process for the transport.

During the chromatography, the flow rate was maintained at 2 ml/min. The flow-through fraction was saved, and the pH was adjusted to around 7.5 with 7 ml of 1 M Tris-HCl buffer, pH 7.5, followed by dialysis against 10 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA. The dialyzed solution was stored at 4 °C and used as a CM-Toyopearl column buffer. The CM-Toyopearl column (Toso) was equilibrated with 180 mM Tris-HCl buffer, pH 7.5. The dialyzed solution was applied to a hydroxyapatite (Wako Pure Chemical Industries) column (8.5 × 50 mm). Adsorbed proteins were eluted with two consecutive linear gradients (10–65 mM potassium phosphate buffer, pH 6.5/110 min and 65–300 mM potassium phosphate buffer, pH 6.5/50 min) at a flow rate of 0.3 ml/min. Fractions of 1.5 ml were collected. Fractions eluted between 30 and 50 mM potassium phosphate were diluted 3-fold with 10 mM Tris-HCl buffer, pH 7.5, and applied to a Mono Q column HR5/5 (Amersham Pharmacia Biotech) equilibrated with the same buffer. Adsorbed proteins were eluted at a flow rate of 0.25 ml/min with two consecutive linear NaCl gradients (0–100 mM/20 min and 100–200 mM/120 min) established in 10 mM Tris-HCl buffer, pH 7.5. Monitoring the absorbance at 280 nm of the effluent, 0.5–1-ml fractions were collected manually. Cyticul proPO was eluted from the column in two fractions: fraction I between 160 and 166 mM NaCl and fraction II between 169 and 175 mM NaCl. These two fractions were rechromatographed separately on the Mono Q column under the same eluting conditions as those described above. In the rechromatography, proPOs in fractions I and II were eluted between 163 and 167 mM NaCl and between 169 and 175 mM NaCl, respectively. The purified proPOs originating from fractions I and II were designated proPO-CS and proPO-CF, respectively.

**Purification of ProPO Isoforms from Hemolymph—**

The collection of hemolymph from silkworm larvae and the first ammonium sulfate fractionation of the hemolymph proteins were performed according to the method of Asakura and Ashida (17). The column chromatography was performed on a Super Q-Toyopearl column, a hydroxyapatite column and first and second chromatography on a Mono Q column in the same way as the cuticular proPO isoforms were purified. In the first Mono Q column chromatography, proPO was eluted in two fractions, fractions III and IV, at NaCl concentrations between 164 and 170 mM and between 173 and 180 mM, respectively. Fractions III and IV were separately subjected to the second Mono Q column chromatography. The proPOs purified from fractions III and IV were designated proPO-HS and proPO-HF, respectively.

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bated. The substrates used were N-acetyl-dopamine (NADA), dopamine (DA), l-DOPA, methylhydroquinone, and hydroquinone. The decrease in absorbance at 265 nm was monitored. The intervals of the monitoring were from 5 to 30 s depending on the kind of substrate and the amount of the enzyme used for the particular assay. From the change in absorbance, the initial rates of the reactions were determined.
formamide and 5 μl of 0.2 M EDTA, pH 5.5, were added. After incubation at 50 °C for 3 min, the mixtures were centrifuged at 10,000 × g for 10 min. The supernatants were used as crude hemolymph proPO fractions. Cuticular proteins were individually extracted from cuticles, and chromatography of each extract was performed using a CM-Toyopearl column chromatography in the above solution except that BSA was substituted forelogex. Fractions giving a single band in electrophoresis were pooled and used for subsequent analyses.

**Determination of Protein**—Protein was determined according to the method of Bradford (27) with a Bio-Rad Protein Assay using bovine serum albumin fraction V as a standard.

### RESULTS

**Purification of Cuticular ProPO Isoforms**—ProPO isoforms present in the cuticles of silkworm larvae at day 5 of the fifth instar were extracted and purified by column chromatography on CM-Toyopearl, Super Q-Toyopearl, hydroxyapatite, and Mono Q columns as described under “Experimental Procedures.” In the first Mono Q column chromatography, they were eluted in two peaks at NaCl concentrations of 0.165 and 0.171 M (Fig. 1A). Each of the isoforms was further purified by the second Mono Q column chromatography, in which they were eluted in a single peak (Fig. 1, B and C). The proPO isoforms eluted at higher NaCl concentrations were designated as proPO-CS and proPO-CF, respectively. Both preparations of the purified proPO isoforms were homogeneous, as judged from the results of PAGE under denaturing and non-denaturing conditions (Fig. 2). A summary of the purification of proPO isoforms is presented in Table I. From cuticles of 70 larvae, 0.282 and 0.374 mg of purified proPO-CS and proPO-CF, respectively, were obtained.

**Isolation of ProPO Isoforms from Hemolymph**—ProPO isoforms present in hemolymph were purified by ammonium sulfate fractionation, heat treatment, and column chromatography on Super Q-Toyopearl, hydroxyapatite, Mono Q columns as described under “Experimental Procedures.” In the first Mono Q column chromatography, the isoforms were eluted in two peaks at NaCl concentrations of 0.168 and 0.175 M (Fig. 1D). The isoforms in the peaks were further purified by Mono Q column chromatography, in which they were eluted in a single peak (Fig. 1, E and F). The proPO isoforms eluted at lower and higher NaCl concentrations were designated proPO-HS and proPO-HF, respectively. Both preparations of the purified proPO isoforms were homogeneous, as judged from the results of PAGE under denaturing and non-denaturing conditions (Fig. 2). From 91.6 ml of hemolymph from the silkworm larvae at day 5 of the fifth instar, 0.472 and 0.882 mg of purified proPO-HS and proPO-HF, respectively, were obtained. Their specific activities were 31.3 × 10⁴ units/mg protein for proPO-HS and 33.7 × 10⁴ units/mg protein for proPO-HF.

**Comparison of Some Molecular and Enzymatic Properties of Native ProPO Isoforms from the Hemolymph and Cuticle**—In PAGE under non-denaturing conditions (Fig. 2B), mobilities of proPO-HS (lane a) and proPO-CS (lane c) were about the same and slightly slower than those of proPO-HF (lane b) and proPO-CF (lane d), which also migrated to about the same positions. Hereafter, the pair of proPO-CS and proPO-HS will be collectively referred to as S-type proPO, and the pair of proPO-CF and proPO-HF will be collectively referred to as F-type proPO (S-type and F-type meaning slower and faster migrating, respectively). Despite the difference in mobilities in the electrophoresis under non-denaturing conditions, all of the proPO isoforms migrated as a single band and did not exhibit any appreciable difference in their mobilities in SDS-PAGE. They migrated to the position corresponding to that of a 71-kDa protein (Fig. 2A).

Both proPO-CS and proPO-CF were determined to be 142-kDa proteins based on their retention times in the Superose 12 column chromatography (data not shown). ProPO-CS and

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2 R. Iwama and M. Ashida, unpublished observation.
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Substrate Specificities of the POs Were Examined at a Fixed Concentration of Several \( \phi \)-Diphenols and \( \phi \)-Diphenols (Fig. 3C).

The phenols were found to serve as better substrates in the oxidation of 4-methylcatechol judging from the results shown in Fig. 3A, and the resulting POs were examined for their abilities to oxidize DA, NADA, and \( \phi \)-DOPA at various concentrations. In Lineweaver and Burk plots, PO-CS and PO-CF exhibited very similar kinetic parameters with each of the three substrates. The plots indicate that the substrates have inhibitory effects on the enzymes at high concentrations. \( K_{m} \) values of the POs for DA, NADA, and \( \phi \)-DOPA were estimated to be about 0.53, 0.8, and 1.9 mM, respectively (Fig. 3B).

Substrate specificities of the POs were examined at a fixed concentration of several \( \phi \)-diphenols and \( \phi \)-diphenols (Fig. 3C). The phenols were found to serve as better substrates in the following order: DA, NADA, \( \phi \)-DOPA, methyldihydroquinone, and hydroquinone. The \( \phi \)-diphenols were barely oxidized under the experimental conditions used in this study. PO-CF and PO-HF exhibited slightly higher activities than did PO-CS and PO-HS toward the \( \phi \)-diphenols. The difference in specific activities was 10–15% in the oxidation of DA. This tendency appears to hold in the oxidation of 4-methylcatechol judging from the data presented in Table I and in the above section, “Isolation of ProPO Isoforms from Hemolymph.”

Activities of the POs were inhibited almost completely by phenylthiourea at a concentration of 1 mM in the oxidation of any of the substrates used in the present study. The effect of \( \phi \)-DOPA on the PO activity was also investigated as described under “Experimental Procedures.” Activities of the four POs in air composed of 20% \( \phi \) and 80% \( \phi \) were about 75% less than those assayed in air composed of 20% \( \phi \) and 80% \( \phi \) (data not shown).

Separation and Analyses of the Subunits of ProPO Isoforms—The four purified proPO isoforms were separated into their subunits by RP-HPLC. Both proPO-CS and proPO-CF were eluted in three peaks (Fig. 4, A and B). On the other hand, proPO-HS and proPO-HF were eluted in only two peaks. In SDS-PAGE, polypeptides contained in the peaks all migrated to a position corresponding to a 71-kDa protein (data not shown). Polypeptides in the peaks originating from proPO-CS were proPO-CS-pI, proPO-CS-pI*, and proPO-CS-pII, in the order of the elution. Similarly, proPO-CF were proPO-CF-pI, proPO-CF-pI*, and proPO-CF-pII. Polypeptides in the peaks of the chromatogram of proPO-HS were proPO-HS-pI and proPO-HS-pII, in the order of the elution, and similarly, those of proPO-HF were proPO-HF-pI and proPO-HF-pII (Fig. 4, C and D). The molecular masses of all of the polypeptides were analyzed by matrix-assisted laser desorption
isoforms migrated. Proteins were stained with CBB. ProPO isoforms applied to lanes a–d were as follows: lane a, proPO-HS; lane b, proPO-HF; lane c, proPO-CS; lane d, proPO-CF. To lane M, marker proteins were applied. The molecular masses of the marker proteins in kDa are indicated at the left of the figure. The molecular masses of native proPO isoforms (proPO-HS, proPO-HF, proPO-CS, and proPO-CF) were determined to be 78,867, 78,857, and 80,080 Da, respectively, and the molecular masses of proPO-HS-pI, proPO-HS-pII, proPO-HF-pI, and proPO-HF-pII were determined to be 78,657, 80,110, 78,681, and 80,050 Da, respectively.

Considering the molecular masses of these subunits and the molecular masses of native proPO isoforms (proPO-HS, proPO-HF, proPO-CS, and proPO-CF), each of which was determined to be 142 kDa as stated in the above section, all the proPO isoforms appear to be composed of two subunits. One of the present authors has reported the similar conclusion that hemolymph proPO is composed of two distinct subunits (12, 25). The conclusion, however, was drawn from the experiment using a mixture of proPO-HS and proPO-HF and, therefore, has yet to be confirmed for each of the hemolymph proPO isoforms. The exact compositions of subunits of each cuticular proPO and the reason why each cuticular proPO isoform was eluted in the two elution profiles (Fig. 5, A and B) were analyzed by mass spectrometry. The results indicated that peptides with the corresponding Arabic numerals in the two elution profiles have the same molecular masses. Several peptides obtained in the RP-HPLC were chosen at random and were sequenced to the 10th residue from their N termini. Observed sequences matched without exception to those in the amino acid sequence deduced from the base sequence of the cDNA, pPO-5 (24) (data not shown). We calculated theoretical molecular mass of the predicted Lys-C fragments that could be produced from the polypeptide with the deduced sequence. All of the observed molecular masses of peptides 1a-16a and 1b-16b matched the expected molecular masses except for those of peptides 1a and 1b, which were 42 Da larger than expected, the difference corresponding to the increase in molecular mass caused by acetylation of the amino group of amino acid at the N terminus (data not shown) (25).

Although peptides corresponding to His88–Lys90 (439.6 Da) and Tyr417–Lys418 (309.4 Da) in the deduced sequence were not identified in RP-HPLC of the digests, these results seem to demonstrate that proPO-HS-pI and proPO-HF-pI have the same primary structures and that their sequences are the same as that encoded by pPO-5.

To study the difference between the structures of proPO-HS-pII and proPO-HF-pII, cDNA (pPO-23) encoding proPO-HS-pII was newly cloned, and the same methodology as that described above was used. In the RP-HPLC of the Lys-C digests of proPO-HS-pII and proPO-HF-pII, two peaks in an elution profile did not have corresponding peaks in another elution profile, as indicated by arrows in Fig. 5 (C and D). The other parts of the elution profiles of the digests were almost the same. The N-terminal sequences and molecular masses of the peptides in the peaks (12c, 12d, 14c, and 14d) were analyzed, and the results are shown in Table II. The N-terminal sequence of 12c seems to correspond to that of Glu417–Glu456 of the polypeptide deduced from cDNA, pPO-17, which has been shown to encode one of the proPO subunits before (25). Peptide 12d had a sequence very similar to that of 12c. However, Pro at the 13th residues of 12c was found to be replaced with Gln in 12d. The same amino acid displacement was found between the putative amino acid sequences deduced from pPO-17 and pPO-23 (Fig. 6). Sequencing of the N-terminal regions of 14c and 14d did not reveal any difference between them (Table II), although the observed sequences were shown to correspond to Met526–Pro556 of the sequences deduced from pPO-17 and pPO-23 (Fig. 6).

Mass spectrometric analysis was performed to further characterize the peptides 12c, 12d, 14c, and 14d. Peptides 12c and 12d were found to have molecular masses of 10,754.8 and 10,802.1 Da, respectively (Table II). These values are very close to the theoretical masses, 10,755.1 and 10,802.1 Da, which were calculated for the peptide fragments spanning from Glu417 to Lys810 of the amino acid sequences deduced from pPO-17 and pPO-23, respectively. Peptides 14c and 14d were found to have molecular masses of 8196.1 and 8208.3 Da, respectively (Table II). These values also match the theoretical masses, 8195.4 and 8210.4 Da, that were calculated for the peptide fragments spanning from Met526 to Lys848 of the sequences deduced from pPO-17 and pPO-23, respectively. These data suggest that the differences in the masses of 12c and 12d and those of 14c and 14d are due to amino acid displacements rather than some kind of modification, although only one amino acid displacement was actually detected by N-terminal sequencing (Table II). As to peptides other than 12c, 12d, 14c, and 14d, the peptides in the corresponding peaks in the two elution profiles in Fig. 5 (C and D) have the same molecular masses. All of the observed molecular masses of the peptides 1c-20c and 1d-20d matched the calculated values of the predicted Lys-C fragments that could be produced from polypeptides with sequences deduced from pPO-17 and pPO-23, respec-
tively, except for molecular masses of 20c and 20d, which were 42 Da larger than the expected masses (data not shown). The difference corresponds to the increase of molecular mass by the acetylation of N-terminal amino group (25). The peptides corresponding to Ile59–Lys62 (469.7 Da), Val144–Lys147 (500.7 Da), and 1-DOPA (panel c) at various concentrations and 0.1 M potassium phosphate buffer, pH 6.0, were prepared. Measurements of oxidation of the substrates were carried out as described under “Experimental Procedures.” The results are presented in Lineweaver-Burk plots. Open circles and solid lines represent the results obtained with PO-CS, and closed circles and dashed lines represent those obtained with PO-HS. Fitted lines were obtained by the least squares method. The velocity \( v \) in the figure is expressed in nmol of oxidized substrate/min. The rates of oxidation of some diphenols by POs was measured at a fixed concentration (0.33 mM) by using ascorbate as described under “Experimental Procedures.” From the observed rates of the decrease of the ascorbate, the rates of oxidation of the substrates were calculated. When DA was used as a substrate, the rates (nmol of oxidized substrate/min/mg enzyme) obtained with PO-CS, PO-HS, PO-CF, and PO-HF were 302.6, 300.9, 335.1, and 348.6, respectively. In the figure, the rate of oxidation of DA by PO-HF was taken as 100.

Table I

| Volume | Protein concentration | Total protein | Total activity | Specific activity | Yield% |
|--------|-----------------------|----------------|---------------|------------------|--------|
| Cuticular extract | 70 | 1.27 | 89 | ND | ND |
| CM-Toyopearl | 90 | 0.731 | 65.8 | 85.7 | 1.3 | 100 |
| Super Q-Toyopearl | 10 | 0.905 | 9.1 | 70.3 | 7.8 | 82 |
| Hydroxyl apttite | 8.5 | 0.451 | 3.83 | 45.6 | 11.9 | 53.5 |
| Mono Q (first) | 2 | 0.305 | 0.61 | 11.5 | 18.7 | 13.4 |
| Fraction II (proPO-CS) | 2 | 0.325 | 0.65 | 17.5 | 26.9 | 20.5 |
| Mono Q (second) | 2 | 0.187 | 0.374 | 9.1 | 32.3 | 10.6 |
| proPO-CS | 1 | 0.282 | 0.282 | 12.6 | 33.7 | 14.8 |
| proPO-CF | 2 | 0.187 | 0.374 | 9.1 | 32.3 | 10.6 |

a The yield was calculated by taking the activity in the CM-Toyopearl fraction as 100%.
b ND indicates that because proPO in the cuticular extract could not be activated by PPAE because of the presence of \( \beta \)-aminophenyl-methanesulfonyl fluoride at a high concentration, the value was not determined.

With the analysis of amino acid sequences of cuticular proPO subunits, it has been observed that the amino acid sequences of proPO-CS and proPO-CF are the same as those of proPO-HS and proPO-HF, respectively, except that same methionine residues are oxidized to methionine sulfoxides (9).

Demonstration of Transport of ProPO from the Hemolymph to the Cuticle—Previously, proPO in the cuticle was suggested to be synthesized in hemocytes (4, 26). In experiments to prove the transportation of proPO from the hemolymph to the cuticle, we utilized the existence of two types of larva, one with only F-type proPO in the hemolymph and the other with both F- and S-type proPO in the hemolymph (12). Larvae were examined individually for the presence of isoforms in their hemolymph and cuticles. Crude hemolymph proPO fractions and crude cuticular proPO fractions were analyzed by PAGE under non-denaturing conditions. It was found that larvae with both...
proPO-HS and proPO-HF in their hemolymph had both proPO-CS and proPO-CF in their cuticles. However, crude cuticular fractions obtained from larvae with only proPO-HF in their hemolymph contained proPO-CF but not proPO-CS (Fig. 7A).

Purified proPO-HS was injected into the hemocoel of each F-type larva at day 0 of the fifth instar as described under “Experimental Procedures.” The larvae that received the proPO isofroms were sacrificed at 24 h after the second injection to prepare crude cuticular proPO fractions. ProPO isoforms in the fractions were analyzed by PAGE under nondenaturing conditions. A proPO band with the same mobility as that of proPO-CS was detected in the crude cuticular fractions (Fig. 7B, lane 6). The intensity of the band seemed to be similar to that observed with crude cuticular proPO fractions obtained from cuticles of FS-type larvae. This clearly shows that the proPO-HS injected into the hemocoel of F-type larva was transported to the cuticle of this larva. ProPO was purified from the cuticles of 12 F-type larvae to which proPO-HS had been injected. The total amount of the purified proPO was about 136 mg. About 53 μg of the total purified proPO exhibited the same electrophoretic mobility under nondenaturing conditions as that of the proPO-CS (Fig. 8A, inset) and was eluted in three peaks in RP-HPLC (Fig. 8B). The retention times of the peaks were the same as those of peaks observed in RP-HPLC of the purified proPO-CS as shown in Fig. 4A. These results indicate that the injected proPO-HS was transported from the hemolymph to the cuticle and that the proPO-HS had been modified to become proPO-CS during the process of transportation or in the cuticle after transportation. We examined the specific activities of proPO isoforms purified from 12 cuticles of F-type larvae to which the purified proPO-HS had been injected. The relative specific activity of the transported proPO-CS was 0.90 if that of proPO-CS copurified in the present experiment is taken as 1.0. There was no apparent significant loss of activity of the injected proPO-HS during the entire process of the experiment.

The modification of some methionyl residues of cuticular proPO to methionine sulfoxides is described in the accompanying paper (9). Interestingly, little of the proPO-CS injected into the hemocoel was transported to the cuticle (Fig. 7B, lane 9).

Northern Blot Analysis of ProPO Transcripts—In a previous study (4), proPO mRNA was not detected by Northern blot analysis in total RNA extracted from epidermal cells. Only cDNA of pPO-17, which encodes proPO-HF-pII, was used as a probe in that study. In the present study, however, pPO-5 was used as a probe in Northern blot analysis, because it was shown to encode proPO-HF-pI and proPO-HS-pI. The results indicated the presence of abundant proPO mRNA in total hemocyte RNA but not in total RNA from the epidermis (Fig. 9). The amount of proPO in the body wall cuticle was shown to increase from days 0 to 5 of the fifth instar. This observation supports our contention that transcripts for proPO must be detected if proPO is synthesized in the epidermal cells.

DISCUSSION

In this study, the properties and the molecular identity of cuticular PO and its zymogen (proPO) of the silkworm, *B. mori*, were investigated. The cuticular PO is often referred to as the injury PO in the literature. In addition to the injury PO, two other types of PO have been shown to be present in the insect cuticle: granular PO and laccase-type PO (10, 28). Different physiological functions have been proposed for these three POs. Granular PO is thought to be responsible for making the body color pattern by synthesizing melanin in the course of normal development, and laccase-type PO is thought to be involved in sclerotization of a newly ecdysed cuticle. The injury PO appears to work in an injured part of the cuticle, possibly by synthesizing cytotoxic quinones and by sealing off the injury. All of these POs have been suggested to be present as inactive precursors, although none of them have been purified (10, 28). The active forms of granular PO and laccase-type PO have been purified from the tobacco hornworm, *Manduca sexta* (29), and the silkworm, *B. mori* (30), respectively. Several attempts to purify the injury PO from orthopteran, dipteran, lepidopteran, and hymenopteran insects have been reported in the literature. All of the attempts, however, failed to give a homogeneous preparation (19, 31–35). The reason for the failure seems to be the adhesive property of the injury PO. As is often encountered with PO from hemolymph, the injury PO appears to form aggregates of itself with progressively higher degrees of association or complexes with other proteins. Thus, the injury PO becomes heterogeneous molecules and has not been able to be purified by conventional techniques.

Insect hemolymph contains PO that is present as a zymogen (proPO) under normal physiological conditions. The precursor forms have been purified from several insect species (12, 17, 28, 36–38). Because the finding of homology between arthropod hemocyanin and proPO in insect hemolymph (25, 38, 39), intensive studies have been carried out, resulting in a rapid accumulation of our molecular biological knowledge on the precursor. Multiplicity of as many as six of the genes encoding proPO subunits in the genome of the mosquito *Anopheles gambiae* has been demonstrated (40), and an upstream region of an *A. gambiae* proPO subunit gene has been shown to have an ecdysone-responsive cis-element and an NF-xB-responsive element. The transcription of the gene was actually shown to be up-regulated by ecdysone (41). Furthermore, the transcript of the gene encoding a protein that possesses two copper binding sites, as observed in proPO or hemocyanin, was detected in the hemocytes of early locust embryos (42). Nascent polypeptide of the locust hemocyanin-like protein has a putative signal sequence for secretion in the deduced sequence, although none of the cDNAs of proPOs that have been isolated contain such signal sequences. These recent observations, however, have not directly contributed to advancing our understanding of cuticular POs, because of the lack of knowledge of the primary structures of cuticular POs. Isolation of cuticular PO and elucidation of the relationship between hemolymph PO and cuticular PO
had not been achieved in previous studies. The importance of such studies for insect physiology was pointed out by Barrett nearly a decade ago (10).

Lai-Fook suggested the presence of a zymogen of PO (injury PO) in 1966 (11). Thirty years later, a new method for the extraction of cuticular proteins from cuticles of the silkworm, B. mori, was developed, and the existence of the zymogen and the cascade for activation of the zymogen was clearly demonstrated for the first time (4). In the present study, we used this method for extraction of the zymogen, and we isolated two homogeneous isoforms of proPO (referred to as proPO-CF and proPO-CS) from the extract (Figs. 1 and 2 and Table I). Previously, silkworm hemolymph was shown to contain two isoforms of proPO (12). We also purified them (referred to as proPO-HF and proPO-HS) for the first time to homogeneity, as judged by the results of SDS-PAGE and PAGE under nondenaturing conditions (Figs. 1 and 2). ProPO-HF and proPO-CF were found to have the same electrophoretic mobilities under nondenatur-

FIG. 5. Peptide mapping of the Lys-C digests of the subunits of proPO isoforms from hemolymph. The subunits of proPO-HS and proPO-HF were obtained as described in the legend of Fig. 4. After, S-pyridylethylolation of the subunits, each subunit was digested with Lys-C. The digests were analyzed in RP-HPLC on an ODS column. Panels showing each chromatogram of the digests and the proPO subunits used for preparing the digests are: A, proPO-HF-pI; B, proPO-HS-pI; C, proPO-HF-pII; D, proPO-HS-pII. Arabic numerals and small letters at the peaks in the figures are to assign the name to the peptide contained in each major peak. Arrowheads indicate the peaks where N-acetylated peptides were detected. Insert, portions (80–120 min) of the chromatograms in C and D are expanded. Details of the experiments are described under “Experimental Procedures.”

TABLE II

| Fragment a | N-terminal sequence b | Mass observed c | Mass calculated d |
|------------|-----------------------|----------------|------------------|
| 12c        | ESAYVRPYTRSELENPGVQ   | 10,754.8       | 10,755.1         |
| 12d        | ESAYVRPYTRSELENQGVQV  | 10,802.1       | 10,802.1         |
| 14c        | MCIEMDFVTVLAGENIQSTESTITIP | 8196.1 | 8195.4 |
| 14d        | MCIEMDFVTVLAGENIQSTESTITIP | 8208.3 | 8210.4 |

a Fragments 12c and 14c originated from proPO-HF-pII. 12d and 14d originated from proPO-HS-pII. The positions where fragments were eluted in the chromatography on an ODS column are shown in Fig. 5 (C and D).

b Determined by Edman degradation.

c Determined by electrospray ionization mass spectrometry.
d Peptides with the same N-terminal sequences as those of 12c, 14c, 12d, and 14d were searched for among postulated lysylendopeptidase digests of the polypeptides with the sequences deduced from cDNA, pPO-5 and pPO-23. The deduced sequences are shown in Fig. 6. S-Pyridylethylations of three cysteines (Cys527, Cys582, and Cys584) were considered in the calculation of predicted masses.
Fig. 6. The deduced amino acid sequence of the subunit proPO-HS-pII of proPO-HS. The amino acid sequence deduced from the cDNA of the clone pPO-23 (upper row) is presented with the sequence deduced from the cDNA of pPO-17 (lower row). cDNAs of pPO-23 and pPO-17 have been concluded to encode proPO-HS-pII and proPO-HF-pII, respectively (see text). Lysine residues are boxed, and the residues where amino acid displacements were detected are shaded. Among the peptides that are predicted to be produced by Lys-C digestion of the polypeptides with the deduced sequences, there are peptides with the same N-terminal sequences and different molecular masses as those observed with the peptides 12c, 12d, 14c, and 14d, which are listed in Table II. The sequences corresponding to the peptides are indicated by horizontal solid lines with arrows at each end together with the calculated molecular masses (the masses with pyridylethylated cysteines are given in parentheses). Amino acid displacements were detected only among the four predicted Lys-C peptides corresponding to 12c, 12d, 14c, and 14d. Open triangles show the cleavage sites by PPAE.

| pPO-23 | pPO-17 |
|--------|--------|
| 1 ADVEFLEEFLFDRENEFLILTSGDENNSFVQTEQLTEDYANNSLNNELNNPGCRADHIP | 1 ADVEFLEEFLFDRENEFLILTSGDENNSFVQTEQLTEDYANNSLNNELNNPGCRADHIP |
| 61 DNIKLELFQELIALLFNLFPDEALNENLVMQYPENVRKQILLCITCAFAPV | 61 DNIKLELFQELIALLFNLFPDEALNENLVMQYPENVRKQILLCITCAFAPV |
| 121 NLIPQELFNYCVAVMHRDITLNRN7FABVPQPEPDLQDVQVTGQAATAVIPDFVPI | 121 NLIPQELFNYCVAVMHRDITLNRN7FABVPQPEPDLQDVQVTGQAATAVIPDFVPI |
| 181 NLIPQELFNYCVAVMHRDITLNRN7FABVPQPEPDLQDVQVTGQAATAVIPDFVPI | 181 NLIPQELFNYCVAVMHRDITLNRN7FABVPQPEPDLQDVQVTGQAATAVIPDFVPI |
| 241 RLQVAFPQRSRLKNSQRRENKRPUPFAYFLSUTSSRQPWHQPSQCGQSWQ | 241 RLQVAFPQRSRLKNSQRRENKRPUPFAYFLSUTSSRQPWHQPSQCGQSWQ |
| 301 NAABGFLYTDIERMNQVENRVEATOGVPLQRTDIDLITGLNMLSALSNPEKL | 301 NAABGFLYTDIERMNQVENRVEATOGVPLQRTDIDLITGLNMLSALSNPEKL |
| 361 VGSIHNSNHSPYTAKMOPKHLYEQQGVIARAKTMOPFYWKYAYIDFXPFVESAY240 | 361 VGSIHNSNHSPYTAKMOPKHLYEQQGVIARAKTMOPFYWKYAYIDFXPFVESAY240 |
| 421 VRFYRTSELEKNQCVRSYSVGPHTQGQPTNLTNKLMLDSDLVNLQGRLSDNPGYFYRTHF480 | 421 VRFYRTSELEKNQCVRSYSVGPHTQGQPTNLTNKLMLDSDLVNLQGRLSDNPGYFYRTHF480 |
| 481 LNYRHFSYRINVNGTSQ5KRTVEFTZETQVSNVPQSDQGC1EMEDFVTPVSNQ550 | 481 LNYRHFSYRINVNGTSQ5KRTVEFTZETQVSNVPQSDQGC1EMEDFVTPVSNQ550 |
| 541 BNNVQ3GSTSSHQTPTPTVEQFRGLAGNQGPNPSTQYFCOQCNQMVMLVHLYQQEAGM | 541 BNNVQ3GSTSSHQTPTPTVEQFRGLAGNQGPNPSTQYFCOQCNQMVMLVHLYQQEAGM |
| 601 PFCQLVWLMINTLTDSDCCDTLOCYTVSSSCICCAPOAEDRAMMPPFPPASSATCC660 | 601 PFCQLVWLMINTLTDSDCCDTLOCYTVSSSCICCAPOAEDRAMMPPFPPASSATCC660 |
| 661 CQFLNRMLGQIDTICLQCNVTHNFNPMM | 661 CQFLNRMLGQIDTICLQCNVTHNFNPMM |

Fig. 7. Identification of proPO isoforms in cuticles of F-type and FS-type larvae (A) and demonstration of the transport of proPO from the hemolymph to the cuticle (B). The types of proPO isoforms were examined by the electrophoresis under nondenaturing conditions. A, crude proPO fractions were prepared individually from larvae. The sources of crude proPO fractions applied were as follows: lane 1, hemolymph of FS-type larva; lane 2, hemolymph of F-type larva; lane 3, cuticle of FS-type larva; lane 4, cuticle of F-type larva. B, purified proPO-HS was injected twice into the hemocoel of an F-type larva and demonstrated of the transport of proPO from the hemolymph to the cuticle (B). The larvae into 12 F-type larvae as described in the legend to Fig. 7B. The larvae were applied as described above except that only the medium for proPO-HS or proPO-CS was injected. Crude cuticular proPO fractions were sacrificed at 72 h after the second injection. ProPO in the cuticles was extracted and purified as described under “Experimental Procedures.” In the purification, a proPO peak in addition to that of proPO-CF was observed in the first Mono Q column chromatography. ProPO in the additional peak and proPO-CF were further purified by rerechromatography on a Mono Q column. The elution profile of the proPO in the additional peak was shown in the figure. The effluent indicated by a horizontal bar was pooled and used as the transported proPO. (A), concentration of proPO; solid line, absorbance at 280 nm; dashed line, concentration of NaCl. Inset, 1 μg each of the transported proPO and the purified proPO-CF were subjected to electrophoresis under nondenaturing conditions. Proteins were stained with CBB. ProPOs and the purified proPO-CF were subjected to electrophoresis under nondenaturing conditions. proteins were stained with CBB. ProPOs applied: lane a, transported proPO; lane b, purified proPO-CF. B, the transported proPO obtained in the chromatography shown in A was subjected to RP-HPLC on an ODS column. The adsorbed proteins were eluted from the column as described in the legend to Fig. 4. Trace c, chromatogram of the transported proPO. Trace d, chromatogram of the purified proPO-HS. Other details are described under “Experimental Procedures.”
PO-CS had almost the same enzymatic properties. It was found that PO-HS and proPO-CF, and proPO-CS, respectively, were examined for HS, PO-CF, and PO-CS, obtained from proPO-HF, proPO-HS, and silkworm cuticles. The resulting active enzymes, PO-HF, PO-HS, and PO-CS, obtained from proPO-HF, proPO-HS, and silkworm cuticles, were examined for their enzymatic properties. It was found that PO-HS and PO-CS had almost the same $K_m$ and $V_{max}$ values for each of the $o$-diphenols such as NADA, DA, and l-DOPA (Fig. 3). Among the three $o$-diphenols, the $K_m$ value was lowest for DA (0.53 mM), and they were 0.80 and 1.9 mM for NADA and l-DOPA, respectively.

All of the POs examined in the present study were inhibited by 75% when their activities were assayed with l-DOPA as a substrate under air composed of 80% CO and 20% O$_2$. The activities of the enzymes were also completely inhibited with 1 mM of phenylthiourea and minimally oxidized p-diphenols (Fig. 3C). Considering their ability to oxidize tyrosine, all of the POs examined in the present study appear to be tyrosinase-type phenoloxidases, not laccase-type phenoloxidases, according to the classification of Keilin and Mann (44).

The transportation of hemolymph proPO to the cuticle has been suggested before in a paper from our laboratory (4). To directly demonstrate this transportation and to elucidate the mechanism of the transportation, we thought that it was important to characterize the molecular properties of proPO isoforms to be transported to the cuticle. Both proPO-HF and proPO-HS each gave two polypeptides in RP-HPLC on an ODS column (Fig. 4). Subunits of proPO-HF were named proPO-HF-pI and proPO-HF-pII, and those of proPO-HS were named proPO-HS-pI and proPO-HS-pII. The peptides in the Lys-C digests of proPO-HF-pI and proPO-HS-pI were analyzed by peptide mapping, electrospray ionization mass spectrometry, Edman degradation. All of the data obtained from these analyses indicated that proPO-HF-pI and proPO-HS-pI were identical polypeptides. Both subunits were proved to have the same amino acid sequence as that deduced from a cDNA clone, pPO-5, reported previously (25). ProPO-HF-pII and proPO-HS-pII were shown to have the same amino acid sequence except at five amino acid residues where amino acid displacements were detected (Fig. 5, C and D, and Fig. 6 and Table II). ProPO-HF-pII was shown to be encoded by a previously known cDNA clone, pPO-17 (25). A cDNA clone, pPO-23, encoding proPO-HS-pII was isolated from a cDNA library of the silkworm hemocytes in the present study. All of the data obtained regarding the four proPO subunits of hemolymph proPO isoforms indicated that the subunits are simple polypeptides without modifications such as glycosylation and phosphorylation. This knowledge of the structure made it easier to determine that modification occurred in cuticular proPO, as described in the accompanying paper (9).

Yasuhara et al. (12) reported that the silkworm proPO in the hemolymph is a heterodimer composed of subunits referred to as proPO polypeptides I and II. The proPO preparation in that study, however, was a mixture of proPO-HF and proPO-HS. Therefore, proPO-HF-pI and proPO-HS-pI in the present study correspond to the proPO polypeptide I, and a mixture of proPO-HF-pII and proPO-HS-pII corresponds to the proPO polypeptide II. In a previous study (12), silkworm proPO in the hemolymph was found to migrate as a doublet in SDS-PAGE. As shown in Figs. 2A and 3A, each proPO isoform migrated as a single band in SDS-PAGE. These results appear to be inconsistent. In reality, however, they are not contradictory, if we consider our finding that proPO gave a doublet in the SDS-PAGE with a separating gel polymerized with a cross-linker, AcrylAide (olefinic derivative of agarose that is no longer commercially available) (12), but gave a single band with separating gels polymerized with bis-acrylamide. In the present study, the separating gel was polymerized with bis-acrylamide.

ProPO transcripts were concluded to be undetectable in total RNA extracted from epidermis of the silkworm larvae at day 2 of the fifth instar, on the basis of the results of Northern blot analyses with a random primed pPO-17 probe (4) and pPO-5 probe (Fig. 9). This was the basis for the speculation that the proPO is transported from the hemolymph to the cuticle. If proPO is labeled with a certain tag and injected into the hemocoel, the recovery of proPO from the cuticle would be direct evidence of transportation. Fluoresceinisothiocyanate or $^{125}$I-labeled proteins have often been used to label proteins. It is possible, however, for these tags to cause conformational changes to the proteins and to introduce artifacts into the experimental results. We took advantage of the presence of two kinds of larvae in the silkworm strain used in the present study. One of them has only F-type proPO in the hemolymph and cuticle, whereas the other has both F-type and S-type proPO isoforms in the hemolymph and cuticle. The larvae with only an F-type isoform are referred to as F-type larvae, and those with F-type and S-type proPO isoforms are referred to as FS-type larvae. To the hemocoel of F-type larvae at the beginning of the fifth instar, purified proPO-HS was injected two times with a 24-h interval, and the larvae were sacrificed to extract proteins from the cuticles at 24 h after the last injection. The extract was analyzed for the presence of S-type proPO by PAGE under nondenaturing conditions. The proPO-HS that had been injected into the hemocoel of the F-type larvae was recovered in their cuticle extracts and showed the same electrophoretic mobility as that of proPO-CS (Fig. 7B, lane 6). This result clearly demonstrates that proPO was transported to the cuticle. Transported S-type proPO was recovered from the F-type larvae and purified to homogeneity (Fig. 7B, lane 6, and Fig. 8A, inset, lane a). No apparent loss of activity occurred during the transportation. Moreover, its elution profile in RP-HPLC was the same as that of proPO-CS as shown in Fig. 4. These results seem to support
the speculation that the proPO-HS artificially introduced into the hemocoel is transported to the cuticle by the same process as that by which endogenous hemolymph proPO is transported to the cuticle under normal physiological conditions. Interestingly, little of the proPO-CS injected into the hemocoel of F-type was transported to the cuticles (Fig. 7B, lane 7). The transport of proPO-HF to the cuticle was not studied because silkworm larva with only S-type proPO isoform in the hemolymph and cuticle was not found in the silkworm strain used in the present study. However, the molecular properties of proPO-HS and proPO-HF were found to be the same except for amino acid replacements at five residues between proPO-HF-PII and proPO-HS-PII, and transcripts of proPO-HF subunits were not detected in total RNA prepared from integumental epidermis as stated above. It therefore seems reasonable to assume that proPO-HF is also transported to the cuticle.

What is the physiological meaning of the presence of proPO isoforms in hemolymph and cuticle? We do not have any definite answer to this question at present. Silkworms with only F-type proPO develop normally, indicating that S-type proPO is not essential molecule for the silkworms as long as they have F-type proPO. Silkworms with only S-type proPO have not been found in the silkworm strain used in the present report. Therefore, it cannot be said with certainty that silkworms with S-type proPO live normally in the absence of F-type proPO, even though molecular properties of F-type and S-type proPO and enzymatic properties of their active forms were determined to be almost the same. In insect species other than the silkworm, B. mori, the presence of at least two kinds of proPO polypeptides has been detected, for example, two in the tobacco hornworm, M. sexta (13), six in the mosquito, A. gambiae (40), and three in the fruit fly, Drosophila melanogaster (FlyBase). The physiological significance of the presence of multiple proPO polypeptides in these organisms also remains to be studied.

The epidermis of the body wall integument has been examined histochemically for the presence of the activity to oxidize DA in a few insect species. The oxidation of DA to melanin was thought to be catalyzed by PO and has been used to detect the enzyme histochemically. Binnington and Barrett (45) used this technique and reported PO activity in the granules of epithelial cells underlying the cuticle of the sheep blowfly, Lucilia cuprina. Locke and Kirshnan (46) observed PO activity in the secretory granules and cis-gorje apparatus of the epidermis of Calpodes ethlius, and they suggested that PO is synthesized in the epidermis and secreted into the cuticle. Because the histochemical techniques used in these studies were not so specific to enable detection of a particular kind of PO, it is possible that laccase-type PO and granular PO might have been detected instead of injury PO. Furthermore, the results should be assessed bearing in mind that the precursor form of PO cannot be detected by the histochemical techniques unless it is converted to the active form during the process of preparing the specimens for the histochemical study. Antibodies raised against granular PO and laccase-type PO have been reported not to cross-react to injury PO (28, 29). Employment of a specific probe such as a monospecific antibody and cDNA would give clear proof of the presence or the synthesis of a particular PO in insect epithelial cells by histochemical techniques.

Arthropods, including insects, have sejate junctions that are considered to be functional equivalents to tight junctions of vertebrates (47). The tight junction has been shown to be a barrier that prevents materials from going through the intercellular space and maintains cell polarity. Insect epithelial cells are highly polarized, and receptor-mediated transport of proteins across cells (transcytotic transport), such as that observed in vertebrates, has been postulated (48). Recently, several of the hemolymph proteins of the tobacco hornworm, M. sexta, have been reported to be transported to the cuticle (2, 8). The transportation was studied by immunocytochemical and immunochemochemical techniques. However, other than their molecular masses, which have been estimated by SDS-PAGE, the molecular properties or the functions of the transported proteins have not been determined. It is not known whether the tobacco hornworm proteins are subjected to any kind of modification during the transportation. As is described above, proPO in the silkworm hemolymph seems to move to the cuticle by transcytotic transportation. By using mono-specific rabbit anti-silkworm proPO/IgG and anti-rabbit goat IgG conjugated to gold colloid, we have detected proPO in the granules of epidermal cells underlying the body wall cuticle of the silkworm. There is no doubt that transcytotic transport of proteins takes place in insect at various epithelial cell layers. Thus, the transportation must be important for the physiology of an insect, but nothing is known about the mechanisms of the transportation. In the present study, proPOs from the hemolymph and cuticle, which are the starting point and destination, respectively, of the transcytotic transportation, were purified to homogeneity and characterized. Modification occurring during the transportation could be inferred from the difference between the chromatographic behaviors of cuticular and hemolymph proPO. It has been proved that modification does not take place during the extraction of proPO from cuticles and subsequent purification. The mechanisms of the transcytotic transportation of macromolecules in insects have yet to be studied. Elucidation of the modification that occurs in the cuticle proPO and how it takes place would be the first important step for advancing our understanding of the mechanism of transcytotic transportation of proteins in insects. In the accompanying paper (9), we report that modification of cuticular proPO involves oxidation of some methionine residues to methionine sulfoxides.

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