Expression of Prophenoloxidase mRNA during Silkworm Hemocyte Development

Kohji YAMAMOTO, Masatoshi YAKIYAMA,* Hiroshi FUIJ,* Takahiro KUSAKABE,** Katsumi KOGA,** Yoichi ASO, and Masatsune ISHIGURO†

Laboratory of Protein Chemistry and Engineering, *Laboratory of Insect Genetic Resources and **Laboratory of Sericultural Science, Kyushu University Graduate School of Bioresource and Bioenvironmental Sciences, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

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Two clones encoding different prophenoloxidase isoforms were amplified by polymerase chain reaction of RNA from the hemocytes of an experimental strain of *Bombyx mori*. The nucleotide sequences of the clones and the deduced amino acid sequences were confirmed to be nearly identical to those of the orthologous clones previously obtained from a commercial race of *B. mori*. Northern blot hybridization using these clones as probes demonstrated that the prophenoloxidase mRNA in the hemocytes is expressed in a stage-specific manner during the final larval instar and pupal stage, showing a peak one day before pupation in males and on the day of pupation in females. A sexual difference was also observed when the content of prophenoloxidase protein in the hemolymph (including hemocytes) was measured by an enzyme-linked immunosorbent assay.

Key words: *Bombyx mori*; hemocytes; prophenoloxidase mRNA

Phenoloxidase (PO: monophenol, dihydroxyphenylalanine; oxygen oxidoreductase; EC 1.14.18.1) catalyzes two consecutive reactions; the hydroxylation of monophenol to o-diphenol and the oxidation of o-diphenol to o-quinone. In insects, PO is known to be involved in cuticular melanization and sclerotization. PO exists in the hemolymph as a precursor protein named prophenoloxidase (pro-PO), which has been purified and characterized extensively from a large number of insects: e.g., the fruit fly *Drosophila melanogaster*, the silkworm *Bombyx mori*, the tobacco hornworm *Manduca sexta*, the cockroach *Blaberus discoidalis*, the wax moth *Galleria mellonella*, and a coleopteran insect, *Holothele diomphalia*. Pro-PO is activated to PO through a limited proteolysis by the prophenoloxidase-activating enzyme (PPAE), which is present in the cuticle and identified to be a serine protease. Organic compounds such as sodium dodecyl sulfate, cetylpyridinium chloride, 2-propanol, dimethyldimethylylammonium chloride (DBMA) are also available as activators, although the activation spectrum depends upon the insect species. Genetic aspects of pro-PO and PO were also investigated. Moreover, pro-PO cDNAs have been cloned and sequenced from the mosquito *Anopheles gambiae*, the fall webworm *Hyphantria cunea*, the coleopteran insect *Tenebrio molitor*, the fruit fly *D. melanogaster*, the tobacco hornworm *M. sexta*, and the silkworm *B. mori*. Many of the species studied so far were found to contain two different pro-PO subunits that are encoded by different genes. In *B. mori*, it was reported that the major synthesis site of pro-PO is the oenocytoid, which is a kind of hemocytes. In this connection it is of interest that pro-PO is a homologue of arthropod hemocyanin. Recently, pro-PO has attracted attention of investigators for its implication in insect immunity, since the activation cascade of this proenzyme is believed to be involved in the defense mechanism against parasite invasion.

Irrespective of these achievements, studies on insect pro-POs from the developmental points of view are scarce except for those dealing with the endocrine control of PO activity in relation to cuticular melanization. Here we analyzed the expression of pro-PO mRNA in the developing hemocytes of *B. mori*, exploiting newly prepared pro-PO cDNA clones as probes. The fluctuation pattern obtained was slightly different from that for pro-PO protein in the hemolymph as analyzed by an immunological method.

† To whom correspondence should be addressed (Tel: +81-92-642-4217; Fax: +81-92-642-3051; E-mail: ishiguro@agr.kyushu-u.ac.jp).

The nucleotide sequences reported in the paper have been submitted to the NCBI with accession numbers AF178461 and AF178462.

Abbreviations: CBB, Coomassie Brilliant Blue R-250; DBMA, dimethyldimethylylammonium chloride; DIG, digoxigenin; t-dopa, L-3-(3,4-dihydroxyphenyl) alanine; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl beta-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PO, phenoloxidase; PPAE, prophenoloxidase-activating enzyme; pro-PO, prophenoloxidase; RT-PCR, reverse transcription-polymerase chain reaction.
Materials and Methods

Insects and preparation of hemolymph and hemocytes. The a80 strain of *B. mori*, maintained in the Silkworm Genetics Division, Institute of Genetic Resources, Kyushu University, was used. Larvae were fed on mulberry leaves. Females and males were separated by observing the imaginal bud on the abdominal surface. Hemolymph was collected on ice in a tube containing solid phenylthioureia to avoid melanization, and centrifuged at 1,000 × g for 15 min at 4°C. The pellet was washed twice with ice-cold 0.75% NaCl and stored at −80°C until used. Alternatively, the hemolymph specimens collected into liquid nitrogen, without centrifugation and without adding phenylthioureia, were lyophilized and the powder was used for the measurement of total pro-PO protein or PO activity.

Reverse transcription-polymerase chain reaction (RT-PCR), cloning, and identification of nucleotide sequences. Total RNA was extracted[20,21] and reverse-transcribed with SuperScript II (Gibco) in the presence of an oligo(dT) primer. Resulting cDNA fragments were amplified in 35 rounds of polymerase chain reaction (PCR). The primers used were designed from the previously reported amino acid sequences at the N- and C-termini and the copper-binding site of the two *B. mori* pro-POs from a commercial race, Kinshu × Showa[20] (see Fig. 1). The sequences of the primers were as follows (underline indicates a 3'-HindIII site and double underline indicates a 5'-BamHI site): 5'-CCAGTCTATG-TCTGACGCCAAGAACAACC-3'(primer1), 5'-CCGGATCCCTACCCCTGCGCAGGCTTGG-3' (primer2), 5'-CCAGCTATAGGCCTGAGCCTTTTTCGAGGCC-3' (primer3), 5'-CCGAGATCTTAAACACGAATGGGAGGTT-3'(primer4), 5'-CCGAGCTTCACCACGTGGCAGTGGACACTCC-3' (primer5), 5'-CCAGACTTCATATTATACCTAGTTGG-3' (primer6). The primers 1/2 and 5/2 were for the pro-PO1 sequence (full-length and partial, respectively) while the primers 3/4 and 6/4 were for pro-PO2 (full-length and partial, respectively). The PCR products were isolated through a 1% agarose gel, extracted with phenol, then digested with *HindIII* and *BamHI*. The resulting fragments were subcloned into a pUC18 vector as described by the manufacturer. Cloned cDNAs were sequenced with a Hitachi DSO-1000 automatic sequencer using a fluorescent-labeled primer cycle sequencing kit (Termo Sequenase) with 7-deaza-dGTP (Amersham).

Northern blot hybridization. Total RNA was extracted by the acid guanidinium thiocyanate phenol chloroform method[22] using Isogen (Nippongene), and samples (8 µg per lane) were separated on 1.2% agarose gel containing 18% formaldehyde and transferred to a Hybond-N+ nylon membrane (Amersham). The membrane was incubated with Digoxigenin (DIG)-labelled probe prepared using a PCR DIG labelling mix (Boehringer Mannheim), then with 0.25 µm disodium 3-(4-methoxyphosphorl,2-dioxyxane-3,2'- (5'-chloro)tricyclo[3.3.1.13.7]decan 4-yI) phenyl phosphate (Boehringer Mannheim) solution, and exposed to Kodak Scientific imaging film.

Preparation of antibody against pro-PO. Pro-PO isoforms were purified from the hemolymph as described[7] and a sample (about 10 µg, a mixture of the three isoforms obtained[7]) emulsified with Freund's complete adjuvant) was injected subcutaneously into a mouse. This was followed by three additional injections at 10-day intervals. The mouse was bled 10 days after the final injection, and the anti-PO serum was recovered.

Measurement of pro-PO content by the enzyme-linked immunosorbent assay (ELISA). Lyophilized hemolymph was dissolved in 10 mM potassium phosphate buffer, pH 7.0, at a concentration of 5 mg protein/ml. The solution was analyzed for the pro-PO protein level by ELISA as described[11] using a 96-well microtiterplate (Falcon). In brief, 50 µg protein/well was reacted with the anti-pro-PO polyclonal antibody, then with anti-mouse IgG conjugated with hors eradish peroxidase. The color intensity was measured at 490 nm after adding o-phenylenediamine and H₂O₂. To get a working curve, various amounts of purified PPO were added to the plate, and the color intensity was measured under the above experimental condition. We could estimate 0.05 µg – 5.0 µg pro-PO in mg of the lyophilized hemolymph or 50 µl of the hemolymph with this ELISA.

Standard assay of pro-PO by PO activity. Pro-PO was assayed for PO activity spectrophotometrically in the presence of a cationic detergent, DBMA, as an activator. The reaction mixture (1 ml) containing pro-PO, 0.05 M potassium phosphate buffer, pH 6.5, 0.4 mM DBMA and 5 mM L-3-(3,4-dihydroxyphenyl) alanine (l-dopa) was incubated at 30°C for 0.5 min. The absorbance at 475 nm was monitored with a Hitachi U-3210 spectrophotometer (Tokyo, Japan). One unit of PO was defined as the amount of active PO capable of producing 1 µmole of dopachrome per min.

Results and Discussion

Cloning of pro-PO cDNAs amplified by RT-PCR and their sequences

Total RNA was extracted from the hemocytes of *B. mori* larvae (the a80 strain) on day 4 of the fifth in-star and amplified by RT-PCR. The primers 1/2 and
3/4 gave fragments of about 2 kbp in size. These were subcloned and the products, named pro-PO1 and pro-PO2 clones, respectively, were analyzed for nucleotide sequence. When the primers 5/2 and 6/4 were used for RT-PCR, fragments with a size of about 1.5 kbp were obtained and these (partial fragments encoding pro-PO1 and pro-PO2, respectively) were used as references.

The full-length pro-PO1 and pro-PO2 clones had open reading frames of 2,058 and 2,082 bp, encoding 686 and 694 amino acid residues, respectively. As to the deduced amino acid sequences (Fig. 1), the similarity between the two pro-PO clones was 50%. The molecular weight and pI expected from the amino acid sequences were 78,728 and 6.2 for pro-PO1, but those were 79,990 and 5.7 for pro-PO2. These data were comparable to those obtained from the two orthologous pro-PO cDNAs, which have previously been cloned using the silkworm race Kinshu × Showa 260 (GenBank D49370, D49371).

However, some difference due to the silkworm origin was found; it was larger in pro-PO1 than in pro-PO2, 3.8% and 0.7% residues, respectively, in terms of amino acid sequence. As shown in Fig. 1, the variation of amino acid residues of pro-PO1 between the two kinds of silkworm was concentrated to the region from 245 to 284 amino acid residues. In this region, 16 out of the 40 residues were different; even a putative N-glycosylation site Asn254-X259-Ser260 seen in Kinshu × Showa was changed. In contrast, only two residues (281 and 282) were different here with respect to pro-PO2. The region in concern does not belong to the domains I to IV, which contain the copper-binding sites and are highly conserved among

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Fig. 1. Comparison of the Deduced Amino Acid Sequences of pro-PO1 (p1) and pro-PO2 (p2) between the a80 Strain and Kinshu × Showa Strain.

The Kinshu × Showa sequences were cited 260 (GenBank, accession nos. D49371 and D49370 for p1 and p2, respectively). Residues of the deduced amino acid sequences are numbered at the left. Double underlines show the sequences from which the PCR primers 1 to 6 were designed. Asterisks, the positions conserved in all four sequences; open and closed triangles, the positions differing within the pro-PO1 and pro-PO2 counterparts, respectively. The following remarks are based on ref. 26: arrow, the site of limited proteolysis during activation by PPAE; roman numerals (I, II, III, and IV), highly homologous regions among pro-POs and hemocyanins from several species (the regions I and II correspond to the copper binding regions A and B, respectively); bold letters, putative N-linked glycosylation sites.
pro-POs as well as hemocyanins from several species. Even within these conserved domains, a80 pro-PO1 had a substitution at residue 229. It is noteworthy that the critical residues Arg11-Phe13, where proteolysis occurs during activation by PPAE, was conserved in all the four sequences. However, the preceding Asn10, which is even conserved in pro-POs from *M. sexta* and *D. melanogaster* (cf. ref. 26), was replaced by His50 in a80 pro-PO1. All Pro-PO isoforms purified from a80 were active when assayed in the presence of an activator. On the basis of these findings, we infer that pro-PO1 tends to receive faster neutral mutations than pro-PO2.

**The expression of hemocyte pro-PO mRNA in comparison with the hemolymph pro-PO content**

To assess the changes in relative abundance of pro-PO mRNA, total RNAs were extracted from hemocyte specimens taken from day 1 of the fifth instar to day 5 after pupation and analyzed by Northern blot hybridization using the pro-PO2 cDNA fragment (2,082 bp) as a probe (Fig. 2). The same developmental patterns as shown in Fig. 2 were obtained when the pro-PO1 cDNA (2,058 bp) was used as a probe (data not shown). The possible variation of the hemocyte concentration in the hemolymph from specimen to specimen could be corrected using an rRNA stain as an internal control. Two major bands occurred until day 2 of the pupal stage; thereafter no bands were detected. The occurrence of dual bands may be related to the fact that the *B. mori* hemolymph has at least two isoforms of pro-PO. However, details remain uncertain and further studies, including that about the degree of cross-hybridization between the pro-PO1 and -2 cDNAs, are needed to clarify the molecular mechanisms under which different transcripts are produced.

![Fig. 2. Expression of pro-PO mRNA in the Developing Hemocytes.](image)

Total RNA from the hemocytes prepared from larvae and pupae of the a80 strain was electrophoresed on agarose gel followed by Northern blot hybridization with pro-PO2 cDNA as a probe. Males (top) and females (bottom) were separately analyzed. Ethidium bromide stain of rRNA is shown as a control for equal loading. 16S rRNA and 23S rRNA from *E. coli* (Boehringer Mannheim) have sizes of 1.7 and 3.5 kbp, respectively. These rRNAs were used as molecular weight standards. Numerals along the abscissa means age in days after the fourth molt or after pupation. S and P represent spinning and pupation, respectively. The bands, smaller than 1.7 kb, found on days 3, 7, and 8 of the male samples and those on days 4, 8, P1, and P2 of the female samples may be artifact products due to degradation.

![Fig. 3. The pro-PO Protein Content in the Developing Hemolymph.](image)

Lyophilized hemolymph specimens (with hemocytes) prepared from larvae and pupae of the a80 strain were subjected to ELISA using the anti-PO polyclonal antibody. About 20 mg of lyophilized hemolymph was prepared from 1 ml of hemolymph. Males (A) and females (B) were separately analyzed. See the legend to Fig. 2 for the numerals and symbols along the abscissa. Bars indicate the standard deviation (n = 3).
There was a sexual difference in the developmental changes of mRNA titer. Males had strong bands on day 3 of the fifth instar and one day before pupation (spinning period, day 8 of the fifth instar), but females had those on day 4 of the fifth instar and on the day of pupation.

Also a sexual difference was marked when the concentrations of total pro-PO proteins in the lyophilized hemolymph specimens prepared from developing larvae and pupae, without removing hemocytes, were analyzed by ELISA, using the anti-pro-PO polyclonal antibody (Fig. 3). The maximum value of 0.43 µg/mg lyophilized hemolymph occurred on day 1 of pupation in males, but that of 0.68 µg/mg lyophilized hemolymph occurred on day 3 after pupation in females. Very similar results were obtained when the pro-PO concentrations in the same specimens were assessed by measuring PO activity in the presence of DBMA (patterns not shown). The PO activity after DBMA activation was maximum (7.54 units/mg lyophilized hemolymph) on day 1 of pupation in males, but it was maximum (9.80 units/mg lyophilized hemolymph) on days 2 to 3 after pupation in females.

There was a gap of one to two days between the peak periods of the mRNA and the protein after the spinning stage (compare Figs. 2 and 3). This finding may have relevance to the previous indication that pro-PO isoforms of B. mori lack a signal peptide to facilitate secretion, suggesting that pro-POs are released by cell rupture from oenocytoids, which have free ribosomes but with no visible rough endoplasmic reticulum. 27

The data presented above clearly showed that the pro-PO proteins are most abundantly present during a few days after pupation in both sexes. This result implies that the activated PO plays a role as a scavenger, removing larval tissues that degenerated during metamorphosis. To substantiate this inference, more detailed investigation about the in vivo activation process is needed, since little or no PO activity was detected in these hemolymph specimens when measured in the absence of DBMA (data not shown).

Interestingly, some of the proteinaceous chymotrypsin inhibitors purified from B. mori hemolymph strongly inhibited PPAE of the same species, although PPAE is a trypsin-type serine protease. 26 A similar situation has been reported for the locust Locusta migratoria. 35 In contrast, the tobacco hornworm M. sexta has a trypsin inhibitor that controls pro-PO activation. 26 These inhibitors may function as regulators for the necessary level of PO activity. Furthermore, the house fly M. domestica was shown to contain a protein that directly inhibits the activity of PO. 26 On the other hand, the above-described differential patterns of mRNA and protein indicate that the hemolymph pro-PO level is controlled both at the transcriptional and post-transcriptional levels including the release from oenocytoids, and these may in turn be regulated by the insect endocrine system. 2627 The sexual difference shown in the fluctuation patterns of mRNA and protein titers may be explained by differential gene expression, since no large variation in growth rate was observed between both sexes. Taken together, these notions indicate that the regulation mechanism of PO activity is more complex than thought before, and more or less varies from species to species.

References


