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Change in phenoloxidase and its precursor during silkworm (a80 strain) development

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The changes in phenoloxidase and its precursor, so–called prophenoloxidase, of the silkworm, *Bombyx mori* (a80 strain) were examined using detection assay with or without detergent to distinguish PO and pro–PO in hemolymph (Yamamoto, *et al.*, 1999). Little or no phenoloxidase activity was detected in hemolymph at the silkworm development during the final larval instar and pupal stage. On the other hand, prophenoloxidase was detected for same time frame. In males, after prophenoloxidase increased slightly at day 4, it decreased to a minimum level at spinning and increased to a maximum level on the day of pupation and decreased gradually to day 5 after pupation. In females, prophenoloxidase decreased gradually to a minimum level on day of spinning and a slight increase was observed at day 8 and it increased to maximum level between day 2 and 3 after pupation and decreased thereafter. Prophenoloxidase mRNA expression was preceded a day or two before, when comparing the change of phenoloxidase mRNA with its product.

INTRODUCTION

Phenoloxidase (PO: monophenol, dihydroxyphenylalanine; oxygen oxidoreductase; EC 1. 14. 18. 1) catalyzes two sequential reactions; the hydroxylation of monophenol to *o*–diphenol and the oxidation of *o*–diphenol to *o*–quinone (Ashida and Yamazaki, 1990). In insects, PO is generally believed to be crucial for the cuticular melanization and sclerotization (Söderhäll, 1982; Hiruma and Riddiford, 1988; Sugunaran *et al.*, 1992).

The existence of a precuesor protein, called prophenoloxidase (pro–PO), was reported from many insects: e.g., the fruit fly *Drosophila melanogaster* (Fujimoto *et al.*, 1993), the silkworm *Bombyx mori* (Yasuhara, *et al.*, 1995; Yamamoto, *et al.*, 1999), the tabacco hornworm *Manduca sexta* (Hall, *et al.*, 1995), the cockroach *Blaberus discoidailis* (Durrant, *et al.*, 1993), the wax moth *Galleria mellonella* (Kopacek, *et al.*, 1995), and a coleopteran insect *Holotrichia diomphalia* (Kwon, *et al.*, 1997). The activation of pro–PO have been shown by the prophenoloxidase–activating factor *in vitro* (Lee, *et al.*, 1998; Jiang, *et al.*, 1998; Satoh, *et al.*, 1999) and by organic compounds such as sodium dodecyl sulfate (Funatsu and Inaba, 1962; Inaba and Funatsu, 1964), cetylpyridinium chloride (Hall, *et al.*, 1995), 2–propanol (Asada, *et al.*, 1993) and dimetylbenzylmyristylammonium chloride (DBMA) (Yamamoto, *et al.*, 1999). Futhermore, the

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cDNA encoding pro–PO have been cloned and sequenced recently from the mosquite *Armigeres subalbatus* (Cho, *et al.*, 1998), the malaria vector *Anopheles gambiae* (Jiang, *et al.*, 1997), the fall webworm *Hypantria cunea* (Park, *et al.*, 1997), the coleopteran insect *Tenebrio molitor* (Lee, *et al.*, 1999), the fruit fly *D. melanogaster* (Fujimoto, *et al.*, 1995), the tabacco hornworm *M. sexta* (Hall, *et al.*, 1995), and the silkworm *B. mori* (Kawabata, *et al.*, 1995; Yamamoto, *et al.*, 2000).

Although many knowleges about pro–PO have been accumulated as described above, the biochemical and genetical aspects of the regulation of PO activity during development is still poorly understood. In housefly, *Musca domestica*, the change of PO activity was examined from larvae to pupae (Funatsu and Inaba, 1962). The authers found that the noticeable change in PO activity occurred at the stage of pupation. Almost the highest PO activity in the homogenate of the final instar larvae disappeared suddenly in the homogenate of the prepupae and then it is appeared again in the homogenate of aged pupae. However, the disappearance of PO activity in the prepupae did not imply that the enzyme vanished because the homogenate of the prepupae exhibited PO activity upon the addition of an anionic detergent such as sodium oleic acid or an extract from the aged pupae. It means that phenoloxidase is present as inactive form in prepupae and there is natural activator, which converts inactive form to active form, in aged pupae.

The fluctuation of PO activity during metamorphosis in the silkworm has never investigated. In order to get a better understanding of the regulatory events involved in the metamorphosis of the silkworm, we analyzed the change in PO activity and pro–PO during developmental stages. The comparison of these fluctuations with that for pro–PO mRNA was also included.

MATERIALS and METHODS

Insects and preparation of hemolymph and hemocytes

The a80 strain of *B. mori* was reared as described previously (Yamamoto, *et al.*, 1999). Females and males were distinguished by the imaginal bud on the abdominal surface. The hemolymph specimens collected into liquid nitrogen, were lyophilized and the powder obtained was used for the determination of total PO activity.

Measurement of PO activity in the crude extract

PO activity was measured spectrophotometrically with or without a cationic detergent, DBMA, as an activator (Yamamoto, *et al.*, 1999). The lyophilized hemolymph (5 mg) from various days was dissolved in 1 ml of 10 mM potassium phosphate buffer (pH 7.0). Fifty μl of this mixture was added to the reaction mixture (1 ml) containing 0.05 M potassium phosphate buffer (pH 6.5), 5 mM L–3–(3,4–dihydroxyphenyl) alanine (L–dopa) and with or without 0.4 mM DBMA. While incubating 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.

Measurement of pro–PO mRNA titer

Northern hybridization was done according to previous paper (Yamamoto, *et al.*,

2000). Level of pro–PO mRNA was assessed by scanning of the electrophoregram by densitometry using NIH image software and replotted.

RESULTS and DISCUSSION

Change in PO and pro–PO at developmental stages

The fluctuation of PO activity during metamorphosis in the silkworm has never investigated. Therefore, we measured the fluctuation of PO or pro–PO and we made better use of detection assay with or without DBMA to distinguish PO and pro–PO in hemolymph.

By using this method, little or no PO was detected, even if the metamorphosis from larva to pupar was occurred (Fig. 1). In housefly, however, the endogeneous PO activity was detected in the homogenate of the final instar larvae, and then it was disappeared suddenly in the homogenate of the prepupae (Funatsu and Inaba, 1962). The presence of active PO in hemolymph is thought to be harmful to the insects health and it is neccesary to activate it during a need. Therefore, insects need to control active PO. Recently, endogeneous PO inhibitors were identified in the housefly, *M. domestica* (Daquinag, *et al.*, 1995) and in the tabacco hornworm, *M. sexta* (Sugumaran and Nellaiappan, 2000) and both were shown to inhibits directly the activity of PO. The PO inhibitor of housefly could inhibit PO activity of silkworm (Yamamoto, *et al.*, 2000, unpublished results). This fact suggests that there might be the strong inhibitor in the silkworm that involves in the regulation system of PO activity, because active PO cannot be detected.

When the changes in pro–PO was determined in hemolymph at developmental stages from fifth larval instar to pupa, pro–PO was detected for thirteen days from larva to pupa (Fig. 1). In males, after pro–PO increased slightly at day 4, it decreased to a minimum level (1.39×10^{-3} unit/mg lyophilized hemolymph) at spinning (day 6) and increased to a maximum level (7.54×10^{-3} unit/mg lyophilized hemolymph) on the day of pupation and decreased gradually to day 5 after pupation. In females, pro–PO decreased gradually to a minimum level on day of spinning (2.41×10^{-3} unit/mg lyophilized hemolymph) and a slight increase was observed at day 8 and it increased to maximum level ($9.80–9.74\times10^{-3}$ unit/mg lyophilized hemolymph) between day 2 and 3 after pupation and decreased thereafter. Pro–PO is always present in this period, whereas no active PO was detected. As shown in Fig. 1, the large amount of pro–PO is stored during a few days after pupation. It is postulated that pro–PO in hemolymph was thought to be stored in case of emergency. Xiao–Qiang *et al.* demonstrated that after injection of bacteria, novel lectin mRNA appeared and lead to stimulate the activation of pro–PO (Yu, *et al.*, 1999).

There is a possibility that some chymotrypsin inhibitors had the ability to inhibit pro–PO activating factor of the same species (Aso, *et al.*, 1994). It was reported some inhibitors found from the locust *Locusta migratoria* (Brehelin, *et al.*, 1991) and *M. sexta* (Saul, *et al.*, 1986) could control pro–PO activation. Pro–PO is always present from larva to pupa, whereas no PO activity is detected (Fig. 1). This result indicates that chymotrypsin inhibitor is likely to control pro–PO activation.

We have recently investigated the developmental changes of mRNA titer in the silkworm, *B. mori* (Yamamoto, *et al.*, 2000). The strong signal of mRNA was detected on day 3 of the fifth instar and one day before pupation in males, whereas females exhibited those on day 4 of the fifth instar and on the day of pupation. Similar results were obtained

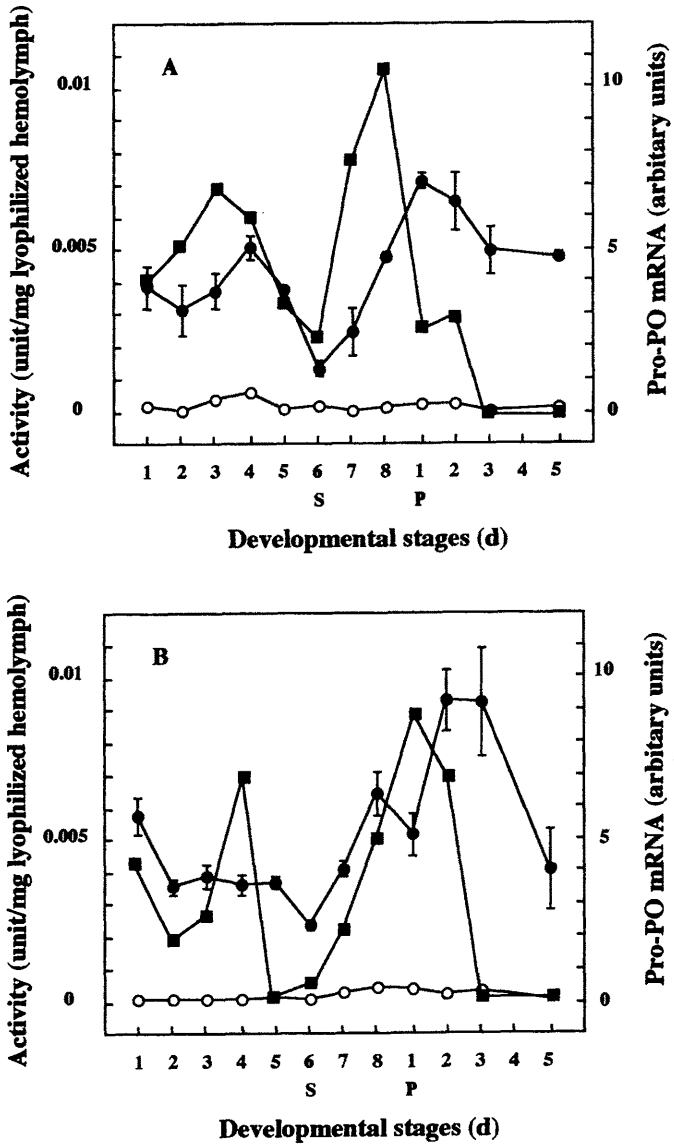

Fig. 1. Developmental changes in pro-PO content evaluated with PO activity and Pro-PO mRNA

PO activity of crude extract was measured after activation with DBMA as described in Materials and Methods. Closed circles represent the assay conducted in the presense of DBMA. Open circles represent the assay conducted in the absense of DBMA. Closed squares represent the level of mRNA. S and P represent spinning and pupation, respectively. Bars indicate the standard deviation (n=3).

when the pro–PO concentrations were measured by enzyme–linked immunosorbent assay.

It is interesting that the fluctuation of pro–PO mRNA of the silkworm was different from that of the fall webworm, *H. cunea* (Park, *et al.*, 1997). They demonstrated that the highest level of pro–PO mRNA was detected in the mid–instar larvae and there was no signal in pupae, adult and egg. There is also the difference between the fluctuation of the silkworm PO and that of housefly PO (Daquinag, *et al.*, 1995). In the housefly, *M. domestica*, by a Western blot detection method using polyclonal antibodies raised against housefly PO, no significant change in PO protein content was observed in the homogenates of the pharate adult compared with that of the homogenates of other stages of aged pupae. They suggested that phenoloxidase inhibitor functions as a regulator of the necessary level of PO.

As shown in Fig. 1, pro–PO mRNA expression was preceded a day or two before, when comparing the change of phenoloxidase mRNA with its product. This finding suggests that the expression of pro–PO could be controlled at transcriptional step. Insect development is known to be regulated by ecdysteroids and juvenile hormones (JH). Possible roles of these hormones in the control of pro–PO expression during development will be studied in detail. The effects of JH on the synthesis of PO were reported. The granular PO that is responsible for cuticular melanization in *M. sexta* larva was controlled by JH (Hiruma, K. and Riddiford, L. M., 1984, 1985 and 1988). Recently, the transcription of PO in *A. gambiae* was regulated by 20–hydroxyecdysone (Martin, *et al.*, 1999). It is also possible for PO synthesis in silkworm to be controlled by 20–hydroxyecdysone at transcription level. Taken together, there are three possibilities of the regulation mechanism of PO activity, that is, transcriptional control, activation control and the inhibition of active PO. In future research, it is important for us to look for PO inhibitor and we will endeavor to understand how this hormone regulates the expression of PO.

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