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Polymorphism of Prophenoloxidase in the Silkworm, *Bombyx mori*

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We reported that the hemolymph of the a80 strain of the silkworm, *Bombyx mori*, included three isoforms of prophenoloxidase, a proenzyme of phenoloxidase. However, their heterogeneity in strains other that a80 has not been investigated. On polyacrylamide gel electrophoresis followed by activity staining for this protein, we discovered that two pro–PO isoforms were revealed in the larval hemolymph of some strains, two strains have three isoforms in their hemolymph, and only hemolymph of a481 strain contain one isoform among silkworm strains we tested. In this paper, we confirmed the polymorphism of prophenoloxidase.

INTRODUCTION

Phenoloxidase (PO: monophenol, dihydroxyphenylalanine; oxygen oxidoreductase; EC 1.14.18.1) catalyzes two successive reactions; the hydroxylation of monophenol to *o*–diphenol and the oxidation of *o*–diphenol to *o*–quinone (Ashida *et al.*, 1990). For insects, PO is thought to be participated in the cuticular melanization and sclerotization (Ashida *et al.*, 1990; Söderhäll, 1982; Hiruma and Riddiford, 1988; Sugumaran *et al.*, 1992). PO occurs in an inactive state, prophenoloxidase (pro–PO), in hemolymph. The enzyme has been purified and characterized from different species: e.g., the fruit fly *Drosophila melanogaster* (Fujimoto *et al.*, 1993), the silkworm *Bombyx mori* (Yasuhara *et al.*, 1995, Yamamoto *et al.*, 1999), the tobacco hornworm *Manduca sexta* (Hall *et al.*, 1995), the cockroach *Blaberus discoidalis* (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 to PO has been shown by the prophenoloxidase–activating enzyme (PPAE), which is present in the cuticle (Dohke, 1973), through a limited proteolysis and identified to be a serine protease (Lee *et al.*, 1998; Jiang *et al.*, 1998; Satoh *et al.*, 1999). Organic compounds such as sodium dodecyl sulfate (Funatsu and Inaba, 1962, Inaba and Funatsu, 1964), cetylpyridinium chloride (Hall *et al.*, 1995), 2–propanol (Asada, 1998), and dimetylbenzylmyristylammonium chloride (DBMA) (Yamamoto *et al.*, 1999) are also available as activators, although the activation spectrum depends upon the insect species. Genetic aspects of pro–PO and PO were also investigated (Asada *et al.*, 1993; Muller *et al.*, 1999). Moreover, pro–PO cDNAs have been cloned and sequenced from the mosquito *Armigeres subalbatus* (Cho *et al.*, 1998),

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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 tobacco hornworm *M. sexta* (Hall *et al.*, 1995), and the silkworm *B. mori* (Kawabata *et al.*, 1995; Yamamoto *et al.*, 2000). In *B. mori*, it was reported that the major synthesis site of pro–PO is oenocytoid, which is a kind of hemocytes (Ashida *et al.*, 1988). 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 responsible for the defense mechanism against parasite invasion (Ashida *et al.*, 1990).

Although much knowledge about pro–PO have been accumulated as described above, their heterogeneity has not been examined so far. Here, we discuss about the polymorphism of pro–PO in the hemolymph of the silkworm, *B. mori*.

MATERIALS AND METHODS

Experimental animal and preparation of hemolymph

The silkworm strains (U901, U902, U903, U904, w213, i40, w23, n12, p21, t70, E24, a481, a48 and a80) maintained in the Silkworm Genetics Division, Institute of Genetic Resources, Kyushu University, were used. Larvae were fed on mulberry leaves. Hemolymph was collected in liquid nitrogen, lyophilized, and stored at -30 °C until use.

Gel electrophoresis

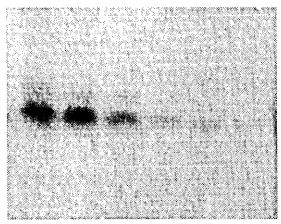
Native polyacrylamide gel (7.5%) electrophoresis (Native–PAGE), followed by activity staining was performed according to Yamamoto *et al.* (1999). Briefly, after pro–PO in the crude samples was electrophoresed on a polyacrylamide gel in the native PAGE, PO activity was visualized by incubating the gel in 5 mM L–dopa and 0.2 mM DBMA in 0.1 M potassium phosphate buffer (pH 6.5) at room temperature. After the appearance of a black band derived from the enzymatic conversion of L–dopa to melanin, the gel was washed with distilled water to remove excess substrate. Proteins were stained with Coomassie Brilliant Blue R–250 (CBB).

RESULTS AND DISCUSSION

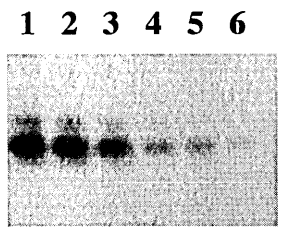
Our results showed the first case of polymorphism of pro–PO. Three electrophoretic variations in pro–PO were observed by Native–PAGE of hemolymph from the larvae of various strains and their representative patterns are shown in Fig. 1. In most of case, the two types of the pro–PO were detected in hemolymph from 11 strains, and the intensity of the former migrated band is higher than that of slower one. Minor difference in the slower band is shown on the result of p21, which means the bands of other strains migrate slightly faster toward the anode than those of p21 strain (Fig. 1). One isoform was contained in hemolymph of a481 strain, whereas three isoforms were detected in hemolymph of a80 and a48 strains.

The difference of a few amino acid residues in pro–POs could not have relation to a diversity of charged isoforms, because we found that each isoform of the silkworm had two subunits of 73 and 74 kDa estimated by SDS–PAGE, when three pro–POs were

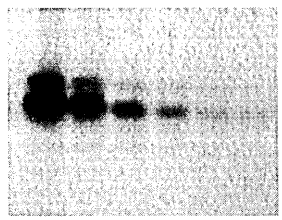
(A)



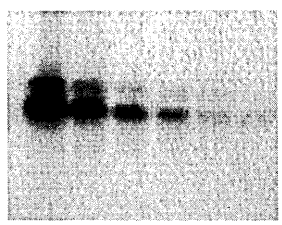
(B)



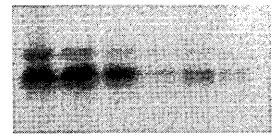
(C)



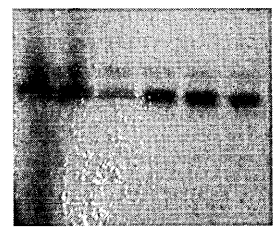
(D)



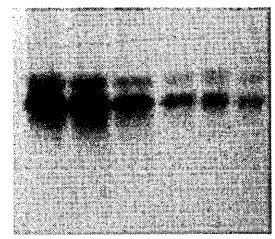
(E)



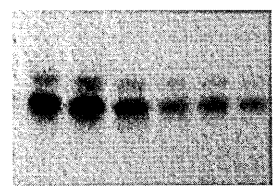
(F)



(G)



(H)



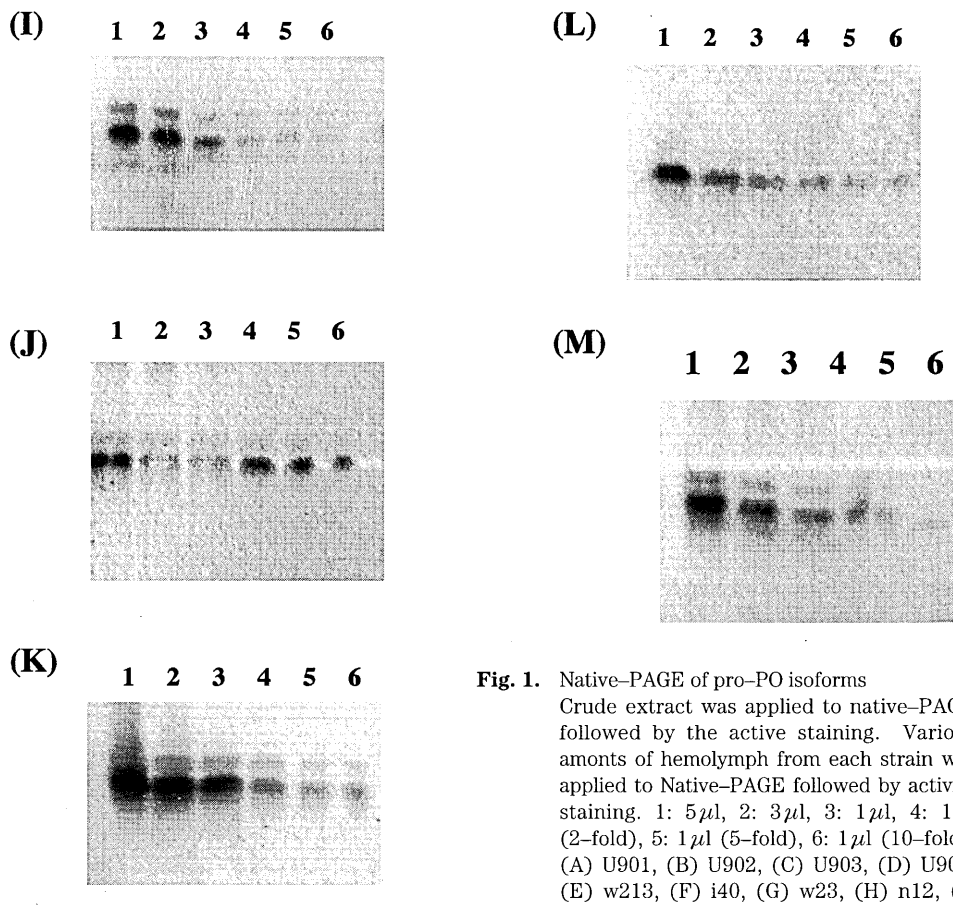


Fig. 1. Native–PAGE of pro–PO isoforms
Crude extract was applied to native–PAGE followed by the active staining. Various amonts of hemolymph from each strain was applied to Native–PAGE followed by activity staining. 1: 5 μl, 2: 3 μl, 3: 1 μl, 4: 1 μl (2–fold), 5: 1 μl (5–fold), 6: 1 μl (10–fold). (A) U901, (B) U902, (C) U903, (D) U904, (E) w213, (F) i40, (G) w23, (H) n12, (I) p21, (J) t70, (K) E24, (L) a481, (M) a48

isolated and characterized (Yamamoto *et al.*, 1999). It was also reported that pro–PO from the Kinshu×Showa strain was resolved in two bands with 71 and 71.5 kDa by SDS–PAGE (Yasuhara *et al.*, 1995). In the freshwater crayfish, *Pacifastacus leniusculus*, pro–PO purified from blood cells was found to be homogeneous on SDS–PAGE and had a molecular size of 76 kDa. (Aspán and Söderhäll, 1991). In the fruit fly, *D. melanogaster*, the molecular size of two pro–POs were estimated to be 77 and 78 kDa by SDS–PAGE (Fujimoto *et al.*, 1993). In the tabacohornworm, *Manduca sexta*, pro–PO exhibited a single band after Native–PAGE and two bands after SDS–PAGE with apparent molecular size of 71 and 77 kDa (Aso *et al.*, 1985). In the previous study, we demonstrated that the difference in the number of potential *N*–linked glycosylation site in pro–PO1 and pro–PO2. It is postulated that the migration of the isoforms depends on the

number of *N*–linked sugar chain. Also, there are three types of this sugar chain such as high mannose type, complex type and hybrid type sugar chain. Therefore, the difference in Asn type sugar chain could be though to have an effect on migration on Native–gel. It is reported that the there is polymorphism of plasma proteins with molecular sizes of approximately 30,000, so–called 30 K proteins, because the primary translation products of 30 K protein mRNA were glycosylated post–translationally in the fat body before being released into hemolymph, resulting in the production of two polypeptides differed slightly in molecular size (Izumi *et al.*, 1981).

Electrophoretic polymorphism has been examined in larval hemolymph chymotrypsin inhibitors and their genetic mechanisms have been studied (Fujii *et al.*, 1996a, b). Also, plenty of inhibitors exist in cocoon appear to be polymorphism (Kurioka *et al.*, 1999). We infer that the expression of protein under control of codominant alleles might result in a mixture of pro–PO isoforms like the hemolymph chymotrypsin/trypsin inhibitors.

We have done the cloning of pro–PO genes from only a80 strain and cloning of pro–PO from other strain is in progress. Detailed study on pro–PO isoforms will provide a clue for understanding why there are many variants of pro–PO in the silkworm, *B. mori*.

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