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Tissue Distribution and Purification of Prophenoloxidase in Larvae of Asian Corn Borer, *Ostrinia furnacalis* Guenée (Lepidoptera: Pyralidae)

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Abstract Using ammonium sulphate precipitation, Blue-Sepharose CL-6B, Phenyl-Sepharose CL-4B, prophenoloxidase (PPO) was isolated and purified from hemolymph of *Ostrinia furnacalis* larvae. This zymogen was a heterodimer, and composed of two subunits with the relative molecular mass ranging from 66.2 kD to 97.4 kD determined by SDS-PAGE. Western blotting and indirect immunofluorescence test showed that PPO was present in integument, hemolymph plasma and cell membrane of granular hemocytes and oenocytoids of *O. furnacalis* larvae.

Key words *Ostrinia furnacalis*; prophenoloxidase; tissue distribution; purification

Phenoloxidase (PO) plays an important role in the defense response to the foreign invaders, e.g. pathogens and parasites, in the formation of melanin as well as in cuticle sclerotization in insects [1]. PO is synthesized as a zymogen, prophenoloxidase (PPO), which can be activated by specific proteolysis. When insects are infected by microorganism, PPO activation is elicited by microbial cell surface components, such as, lipopolysaccharide (LPS), peptidoglycans, β-1,3-glucose [2].

To further clarify the molecular mechanism of PO in insect physiology and biochemistry, a detailed study on this enzyme is necessary. However, due to the instability and rapid loss of the activity of this enzyme during the purification, more attention has been paid to the investigation of PPO. So far, PPO has been purified and characterized from only a small number of insect species including lepidopterans, dipteran, cockroaches and locusts [3].

The Asian Corn Borer *Ostrinia furnacalis* is one of major crop pests, and has been used as a model insect for the investigation of the physiological interaction between this insect species and a polyembryonic parasitoid *Macrocentrus cingulum* Brischke [4]. Obviously, it is desirable to obtain more information dealing with the physicochemical properties of *O. furnacalis* PPO. We report here the tissue distribution and purification of PPO from hemolymph of *O. furnacalis* larvae.

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Materials and Methods

Insects

O. furnacalis larvae were reared at (25 ± 1) °C and > 90% R.H. with a 14:10 h light:dark photoperiod. The larvae were fed on an artificial diet as described by Zhou *et al*. [5]. The larval instars were selected by measuring the head-width.

Collection of hemolymph, hemocytes, fat body and integument

Hemolymph and hemocytes from 5th-instar *O. furnacalis* larvae were collected by using the method of Charalamibidis *et al*. [6]. Collection of integument and fat bodies from 5th-instar *O. furnacalis* larvae as well as homogenization of hemocytes, integument and fat bodies were performed as described by Charalamibidis *et al*. [7].

Extraction of membrane protein from hemocytes

Hemocyte membrane and cytosol protein were separated with a procedure described by Cui *et al*. [8].

Standard assay of PPO

To activate PPO, a reaction mixture (1 ml) containing 2 mM dopamine, 50 mM sodium phosphate buffer (pH 6.0) and enzyme protein (5–10 μg) was incubated at 25 °C and the increase in absorbance at 490 nm (A_{490}) associated with the production of dopaminechrome was continuously monitored after activating PPO by the addition of 10 μl 10% CPC (cetylpyridinium chloride). One unit of PO was defined as an increase of 0.001 in A_{490} per min.

Enzyme purification

All steps were performed at 4 °C in cacodylic acids buffer (20 mM $CaCl_2$, 10 mM Na_2CAC, pH 6.5), unless otherwise noted. Hemolymph (8 ml) was first diluted into 2:1 with the CAC buffer. The saturated ammonium sulphate solution was carefully added to hemolymph until reaching a saturation of 40%. The precipitate was spun down by centrifugation (12,000 *g*, 10 min, 4 °C), dissolved in 500 μl of 20 mM sodium cacodylate (pH 6.5). The protein was dialyzed in 2000 ml CAC buffer overnight at 4 °C, and then applied to a Blue Sepharose CL-6B column (1.0 cm×10 cm) previously equilibrated with CAC buffer. The column was eluted with elution buffer (100 mM $CaCl_2$, 10 mM Na_2CAC, pH 6.5) at a flow rate of 1.5 ml/min. Fractions containing PPO from three simultaneous Blue Sepharose CL-6B chromatography were pooled (about 1.5×20 ml) and immediately applied to a Phenyl-Sepharose CL-4B column (0.8 cm×12 cm) that was equilibrated with CAC buffer. The column was washed with distilled water at a flow rate of 1.5 ml/min until the absorbance of fractions at 280 nm returned to zero. The fractions with PO activity were dried in a Heto FD3 Model Vacuum Cold Dryer. The purified proenzyme was stored in the presence of 0.02% NaN_3 at –80 °C until use.

Electrophoresis

SDS-PAGE was carried out in 10% polyacrylamide in non-reducing conditions according to the method of Laemmli [9]. Native PAGE was performed according to Tyagi *et al*. [10] with 6% polyacrylamide gel. The proteins were stained with Coomassie brilliant blue. Protein concentrations were determined by the method of Bradford using bovine serum albumin (SERVA) as a standard.

Antibody generation and Western blotting

The polyclonal antibody against *O. furnacalis* PPO was prepared using a young adult rabbit (New Zealand White, 4 kg). Western blotting analysis of purified protein was performed using anti-*O. furnacalis* PPO serum as primary antibody according to the method of Sambrook *et al*. [11].

Indirect immunofluorescence assay (IIFA)

IIFA analysis of PPO binding to the hemocytes of *O. furnacalis* larvae was performed using the method of Cui *et al*. [8]. The hemocytes were treated in turn with: (1) rabbit antiserum against *O. furnacalis* PPO at a dilution of 1:2000; and (2) fluorescein isothiocyante (FITC)-labelled goat anti-rabbit IgG (1:3000) (Amershan Life Science). Controls used were the deletion of either the primary or the secondary antibodies. The hemocytes were examined under a Zeiss Axioskop microscope with epifluorescent illumination at 1000× magnification.

Results

Tissue distribution of PPO in *O. furnacalis* larvae

Western blotting analysis of protein lysate from fat body, integument homogenate, serum, and hemocyte lysate of *O. furnacalis* larvae using anti-*O. furnacalis* PPO serum revealed that PPO was present in all above tissue preparations except in fat body (Fig. 1).

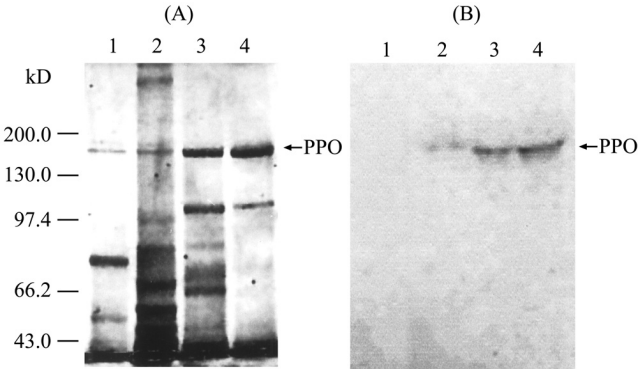

Fig. 1 Detection of PPO in various tissues of *O. furnacalis* larvae

(A) Native PAGE of PPO. 1, fat body; 2, serum; 3, hemocyte lysate; 4, integument. (B) Western blotting of native PAGE of PPO in various tissues, using anti-*O. furnacalis* PPO serum as primary antibody. 1, fat body; 2, integument; 3, hemocyte lysate; 4, serum.

Cellular localization of PPO

Western blotting analysis demonstrated that the purified PPO showed a positive reaction with anti-*O. furnacalis* PPO serum. The isolated membrane protein of hemocytes from *O. furnacalis* larvae was also immunoreactive,

whereas the debris of hemocytes after removing the membrane protein was negative (Fig. 2). This result indicated that PPO was present in the membrane of hemocytes but absent in debris of hemocytes of *O. furnacalis* larvae. When indirect immunofluorescence test was used to examine what types of hemocytes containing PPO it was found that only the surface of oenocytoids and granular hemocytes was clearly labelled (Fig. 3).

Purification process of PPO from hemolymph of *O. furnacalis* larvae

A typical result of PPO purification from plasma was shown in Table 1. The purification procedure yielded 0.29 mg of PPO from a starting sample of 8 ml hemolymph containing about 276.30 mg total protein. PPO was puri-

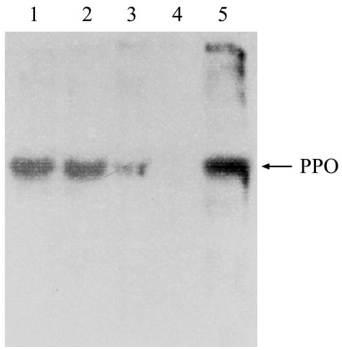

Fig. 2 Immunoblotting analysis of PPO of *O. furnacalis* larvae

1, the isolated membrane protein of hemocytes (5 μg); 2, serum (10 μg); 3, integument (10 μg); 4, debris of hemocytes after removing of membrane protein (10 μg); 5, purified PPO (0.5 μg).

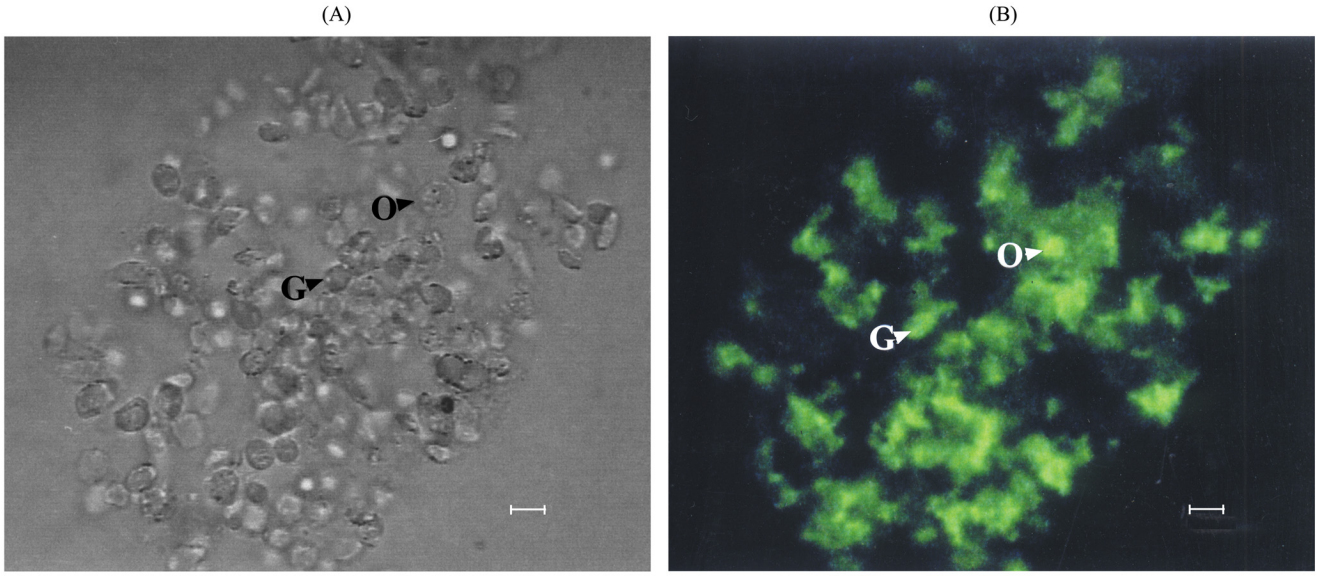

Fig. 3 Indirect immunofluorescence assay of PPO on hemocytes of *O. furnacalis*

(A) Light microscopical image. (B) Fluorescent microscopical image. G, granular hemocytes; O, oenocytoids. Scale bars=15 μm.

Table 1 Purification of PPO from hemolymph of *O. furnacalis

Step	Total amount of protein (mg)	Specific activity (U/mg)[b]	PO activity (U)[a]	Recovery (%)	Purification (fold)
Hemolymph	276.30	30.40	8399.50	100.00	1.00
$(NH_4)_2SO_4$	76.22	88.16	6719.60	80.00	2.90
Blue-Sepharose CL-6B chromatography	5.32	1215.66	6467.30	76.99	39.99
Phenyl-Sepharose CL-4B chromatography	0.29	9847.59	2855.80	33.99	323.93

*PO activity was determined by the activation of PPO after each purification step with CPC (cetylpyridinium chloride). [a] expressed in ΔA_{490}/min; [b] expressed in ΔA_{490}/min for per mg of protein.

fied 323.93-fold with a recovery of 33.99%. Native PAGE on 6% gel of the purified PPO showed one single band, suggesting that this zymogen was purified to homogeneity [Fig. 4(A)]. The protein eluted from Phenyl-Sepharose CL-4B chromatography was also verified in Western blotting analysis using anti-*O. furnacalis* PPO serum [Fig. 4 (B)]. The finally purified PPO exhibited two closely moving bands on 10% SDS-PAGE. Their estimated molecular masses were in a range of 66.2 kD to 97.4 kD (Fig. 5).

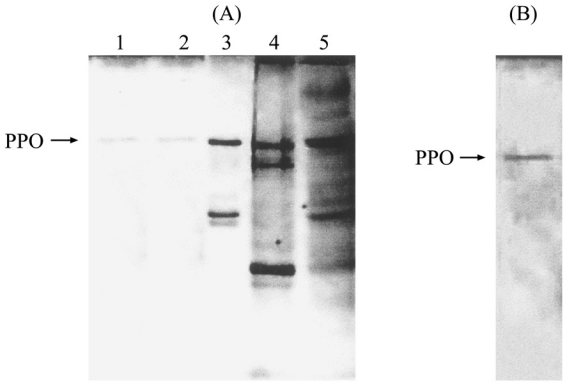

Fig. 4 Native PAGE (6%) of different fractions in the process of purification (A) and Western blotting detection of purified protein from fractions of Phenyl-Sepharose CL-4B chromatography (B)

1 and 2, the fractions of Phenyl-Sepharose CL-4B chromatography (0.5 μg); 3, the fractions of Blue-Sepharose CL-6B chromatography (2 μg); 4, the precipitation of ammonium sulphate (3.5 μg); 5, serum (10 μg).

Discussion

Western blotting analysis using anti-*O. furnacalis* PPO serum detected one protein band from serum, hemocytes lysate and integument extract of *O. furnacalis* larvae, but not from fat body. This result along with those reported in other insect species indicated that the insect tissues were bathed in or surrounded by this enzyme zymogen and its activating cascade [12]. Since *O. furnacalis* PPO was only detectable in the isolated membrane of the hemocytes, it suggested that *O. furnacalis* PPO might be synthesized in hemocytes, and transported into hemolymph plasma. In the mosquito *Armigeres subalbatus* it was proposed that PPO was released from granulocytes by cell rupture, synthesized or stored in granulocytes [13]. Semiquantitative RT-PCR analysis of RNA from heads, thoraxes and abdomens of *Anopheles stephensi* showed similar PPO expression pattern in these body parts. RT-PCR analysis of RNA isolated from different tissues of the female mosquito showed that Ans-PPO expression was detected in

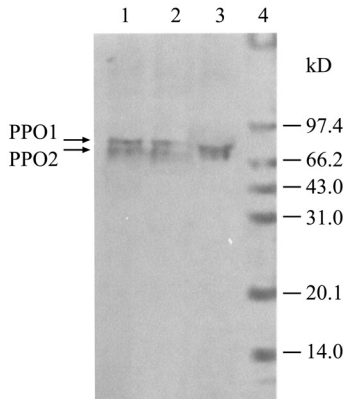

Fig. 5 SDS-PAGE of the purified PPO

1, purified PPO (1 μg); 2, purified PPO (2 μg); 3, purified PPO (3 μg); 4, marker proteins (2 μg of each).

hemocytes, carcasses (after removal of midgut and ovaries) and ovaries. However, hemocytes were the primary source of PPO synthesis [12]. In *Anopheles gambiae* and *A. stephensi*, the presence of PO activity in the midgut epithelium and salivary glands further suggested the existence of a transport mechanism for PPO [14, 15].

In lepidopteran insects, five types of hemocytes are classified, namely prohemocyte, granular hemocyte, plasmatocyte, spherule hemocyte and oenocytoid. However, our result indicated that only granular hemocytes and oenocytoids were possibly associated with PPO synthesis in *O. furnacalis*. In *M. sexta*, it was reported that oenocytoid was the major site of PPO synthesis [16], whereas in *Procambars clarki* granular hemocytes and semigranulocytes were considered as the source of PPO production [17].

Since the immunoaffinity chromatography was proven ineffective to purify PPO [18], the conventional chromatographic method was employed by several investigators [18, 19]. The similar method was used in this study to purify PPO from hemolymph of *O. furnacalis* larvae. The finally eluted PPO from Phenyl-Sepharose CL-4B chromatography was 323.93-fold purified (Table 1). This indicated that our protocol using Blue-Sepharose CL-6B followed by Phenyl-Sepharose CL-4B was effective in processing the purification of PPO. Native PPO of *O. furnacalis* larvae showed one single band in native PAGE, whereas under non-reducing condition of SDS-PAGE it consisted of two subunits with the relative molecular mass ranging from 66. 2 kD to 97.4 kD. This result agreed with those reported in other insect species [1,18,20]. More accurate estimation using mass spectrometry revealed that the molecular mass of the two *M. sexta* PPO subunits was 78 kD and 80 kD,

respectively [16], which was close to the estimation from cDNA sequence. To date, at least two PPO isoforms were detected in lepidopteran and dipteran insects, whereas only one was identified from orthopteran insects [21]. These data were consistent with the hypothesis that a PPO gene duplication occurred after the divergence of holometabolous and hemimetabolous insects. Since PPO was purified only from a few arthropod species, more data were needed for better understanding the molecular evolution of this proenzyme encoding gene.

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