Effect of the endoparasitoid *Campoletis chlorideae* on phenoloxidase activity in *Helicoverpa armigera* hemolymph

YIN Lihong, WANG Chenzhu & QIN Junde

State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China

Correspondence should be addressed to Wang Chenzhu (e-mail: czwang@panda.ioz.ac.cn)

Abstract **Endoparasitoid wasps can develop inside permissive host due to their ability to overcome or to evade the host's cellular and humoral immune response. Oviposition of *Campoletis chlorideae* (Hymenoptera: Ichneumonidae) in larvae of *Helicoverpa armigera* (Lepidoptera) was accompanied by inhibition of phenoloxidase (PO) activity and melanization reaction in host hemolymph *in vitro*. PO activity in host plasma was decreased about 83% 48 h post parasitization. A similar result was found when the host insect was injected with 0.5 wasp equivalent calyx fluid. This indicated that the calyx fluid was concerned with suppression of PO activity after parasitization. Furthermore, the prophenoloxidase (proPO) in host haemocytes could be activated by bovine trypsin in unparasitized insects, while it could not be activated in parasitized or calyx fluid-injected host. The results suggested that inhibition of PO activity by parasitization was related to the calyx fluid of *Campoletis chlorideae*, and the components of calyx fluid (eg. polydnaviruses) perhaps suppressed the expression of proPO in hemolymph or accelerate the degradation of proPO.**

Keywords: insect immunity, phenoloxidase, prophenoloxidase, endoparasitoid, calyx fluid.

Foreign objects entering an insect are recognized as non-self leading to the induction of a variety of humoral and cellular immune reactions. Humoral immunity is carried out by some soluble hemolymph proteins, whereas cellular immunity includes phagocytosis, encapsulation and nodule formation to the foreign objects[1]. Melanization of the invading organism due to the phenoloxidase activity of the host is often associated with these processes. Phenoloxidase is considered as the essential component of the insect's humoral defense reactions, and usually exists as an inactive proenzyme in the hemolymph. The activation of prophenoloxidase (proPO) can be achieved by a limited proteolysis by serine protease. Activated PO catalyzes tyrosine and DOPA to melanin via toxic quinone intermediates[2].

Since insect immune systems are able to deal effectively with the invading objects, endoparasitoids must have evolved strategies to circumvent the defence reactions of their habitual hosts. Ichneumonid and Braconid endoparasitoid ovarian secretions contained polydnavirus (PDV), venom and soluble proteins. The secretions are released into the host together with the parasitoid egg and play an important role in protecting the egg from being encapsulated in host body cavity[3,4]. Gene expression of PDV in the calyx fluid of female wasp's ovary leads to immunosuppression to host and to successful parasitization of the wasps. It was reported that hemolymph PO activity in *Heliothis virescens* and *Manduca sexta* could be inhibited by PDV from ichneumonid *Campoletis sonorensis* and braconid *Cotesia congregata* respectively[5,6]. In this note we report that hemolymph PO activity of *Helicoverpa armigera* was significantly decreased at the early stage of parasitization of *Campoletis chlorideae* or injection with its calyx fluid, which suggested that polydnaviruses might exert an inhibitory effect on host hemolymph PO activity during parasitization. The possible mechanism was discussed.

1 Materials and methods

(ⅰ) Insects. *H. armigera* larvae were reared on the artificial diet as described by Wang and Dong at (27±1)℃ and a 15-h light (L)∶9-h dark (D) photoperiod[7]. *C. chlorideae* was reared in *H. armigera* larvae as described in ref. [8]. Late 2nd- or early 3rd-instar *H. armigera* larvae were attacked by mated female wasps for one or two times for oviposition. The parasitized host larvae were kept individually at (27±1)℃ and a 12L∶12D photoperiod. The adult wasps were fed with 10% honey-water solution.

(ⅱ) Parasitization and calyx fluid injection. Early 3rd-instar host larvae were attacked by female wasps for one or two times. Ovaries from several female wasps were dissected in Ringer's solution by gently pulling the ovipositor from the abdomen. The calyces were removed and punctured using forceps and the contents were allowed to diffuse into Ringer's solution. After centrifugation for 3 min at 800 *g*, 1 μL supernatant (a dose of 0.5 wasp equivalent of calyx fluid) was injected into each late 3rd- or early 4th-instar larva. As control, 1 μL Ringer's solution was injected into each host larva.

(ⅲ) *In vitro* melanization reaction. In order to access the capacity of melanization reaction in the whole hemolymph of the host, cohorts of 15 host larvae were selected at disignated times post parasitization or injection. Hemolymph samples from unparasitized, parasitized or injected larvae were collected on a glass slide by puncturing the larval proleg with a sterile insect pin. The drop of undiluted hemolymph was left for 20 min at ambient room temperature. A change in coloration of the hemolymph, from opaque or green to brown-black, was recorded as normal melanization, whereas the maintenance of the previous color or change in an intermediate color was considered as reflecting an inhibition of melanization[9].

(iv) Preparation of plasma and hemolymph lysate supernatant (HLS). Cohorts of 6—8 larvae were selected from unparasitized, parasitized or injected *H. armigera* larvae. Hemolymph was collected in 0.5 mL AC-buffer (62 mmol/L NaCl-100 mmol/L glucose-10 mmol/L EDTA-30 mmol/L trisodium citrate-26 mmol/L citrate acid, pH 4.6) in the same way as explained above. The diluted hemolymph was centrifuged at 800 *g* for 5 min, the supernatant was dialyzed against 1 L of 0.01 mol/L sodium cacodylate-buffer, pH 7.0, for 1 h at 4℃ to remove AC. The plasma was then assayed for PO activity after the addition of $CaCl_2$ to 5 mmol/L. The haemocyte pellet was washed twice with cac-buffer (0.01 mol/L sodium cacodylate-buffer, pH 7.0, containing 5 mmol/L $CaCl_2$) and 0.25 mol/L sucrose, then suspended in 200 μL cac-buffer before sonification. The cell homogenate was then centrifuged at 2800*g* for 15 min and the resulting supernatant was used as a source of the proPO system and designated haemocyte lysate supernatant (HLS)[10].

(v) Assay for phenoloxidase activity. Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA). The plasma (50 μL) was placed in a microwell plate and pre-incubated with an equal volume of cac-buffer for 30 min at 25℃ before 50 μL of L-DOPA (3 g/L) was added. After a 10-min incubation at 25℃, the optical density was read at 490 nm using a Microplate reader (BIO-RAD). The specific activity was defined as the change of absorbance per min per mg of protein[11].

(vi) Activation of proPO by bovine trypsin. The HLS (50 μL) was pre-incubated with an equal volume of bovine trypsin (2 mg/mL), or in the case of controls, with cac-buffer for 30 min at 25℃ before 50 μL L-DOPA (3 g/L) was added. The reaction was allowed to proceed for 10 min at 25℃, after which the absorbance at 490 nm was measured every 10 min by Microplate reader (BIO-RAD)[12].

2 Results

(i) Effect of parasitization and calyx fluid injection on haemolymph melanization. The hemolymph melanization reaction of *H. armigera* was significantly inhibited after parasitization and calyx fluid was injected (fig. 1). Melanization was observed in 100% of samples collected from unparasitized insects when exposed to air for 20 min, whereas melanization rate was dramatically decreased in samples collected from parasitized insects. At 36 and 72 h post parasitization, the percentage of hemolymph melanization was decreased to 72.7% and 40% respectively; at 36 and 72 h after calyx fluid was injected, that was decreased to 80% and 62.8% respectively. Anyway the proportion of melanization reactions was slightly increased at 96 h post parasitization or injection. In order to exclude the possibility that the inhibition of hemolymph melanization by injection of calyx fluid was caused by the hurt to larva cuticle, we injected Ringer's solution as control. Almost 100% of samples in the control were melanized.

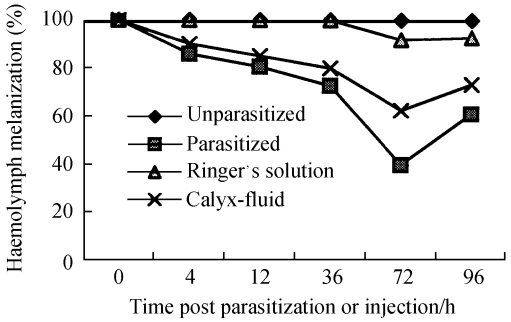

Fig. 1. Haemolymph melanization reaction *in vitro* at various times post parasitization or injection.

(ii) Effect of parasitization and injection on phenoloxidase activity in hemolymph. Using a citrate-EDTA buffer as an anticogulant we found that it was possible to isolate intact haemocytes from the insects[10]. Since PO was released into plasma after being synthesized in haemocytes[10,13], we assayed the PO activity in plasma (table 1). It showed that PO activity was significantly inhibited ($P<0.05$) at 48 h post parasitization, while it was slightly recovered at 96 h post parasitization. Injection of calyx fluid at a concentration of 0.5 female equivalent had the similar effect of parasitism on PO activity.

Table 1 Comparison of PO activity in plasma of *Helicoverpa armigera* larvae in different treatments (A_{490}/min per mg protein)[a)]

	48 h	96 h
Unparasitized	0.337 ± 0.049^{a}	0.324 ± 0.052^{b}
Parasitized	0.057 ± 0.002^{c}	0.155 ± 0.011^{c}
Ringer's solution-injected	0.203 ± 0.029^{b}	0.458 ± 0.033^{a}
Calyx fluid-injected	0.063 ± 0.008^{c}	0.203 ± 0.019^{bc}

a) The data in the table was mean ± SE (n=3). Means followed by the same letter within a column were not significantly different (P = 0.05; Duncan's multiple range test).

(iii) Activation on proPO by bovine trypsin. For the proPO system is mainly localized in the haemocytes of insects[10], we provided a crude proPO preparation with haemocyte lysate supernatant (HLS) for detection of proPO activation by bovine trypsin[14]. As shown in figs. 2 and 3, PO activity was significantly increased when activated by trypsin in samples of unparasitized and Ringer's solution-injected larvae, whereas it was slightly decreased in parasitized and calyx fluid-injected samples (figs. 4 and 5).

3 Discussion

The first challenge an endoparasitoid encounters in its habitual hosts is the immune response of the host: eggs of endoparasitoids laid in hemocoel of host insects face a potential encapsulation reaction by haemocytes of host, and the encapsulation is often followed by melanization.

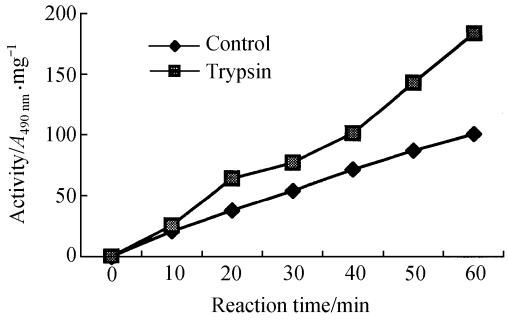

Fig. 2. ProPO activated by bovine trypsin in unparasitized haemolymph.

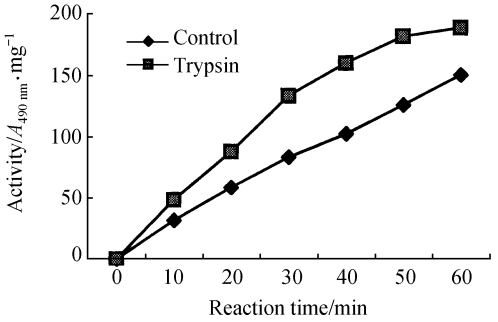

Fig. 3. ProPO activated by bovine trypsin in Ringer's solution-injected haemolymph.

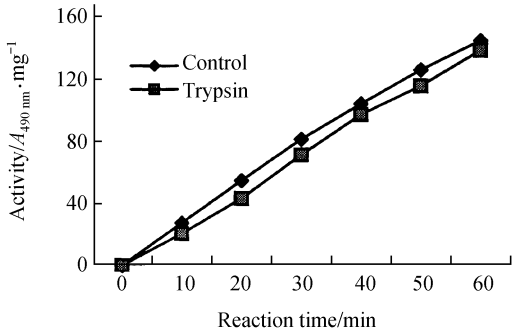

Fig. 4. ProPO activated by bovine trypsin in parasitized haemolymph.

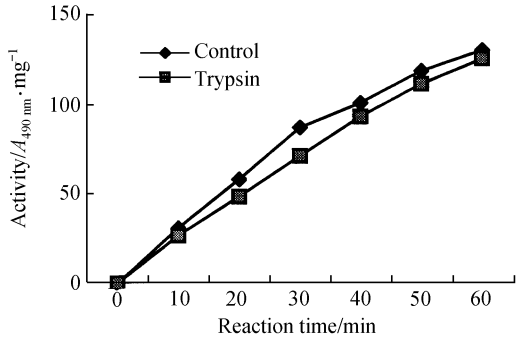

Fig. 5. ProPO activated by bovine trypsin in calyx fluid-injected haemolymph.

In the long process of evolution, endoparasitoids have evolved a variety of strategies to circumvent the immune reaction of their habitual hosts. The ability of endoparasitoid to control host immunity is well reflected in regulating host hemolymph phenoloxidase activity. Our experiments showed that phenoloxidase in *H. armigera* was inhibited when it was parasitized by *Campoletis chlorideae* or injected with calyx fluid. PO activity and the percentage of hemolymph melanization post parasitization or post injection was significantly lower than the respective controls, and this kind of inhibition will provide a favorable condition for the early development of eggs in host hemocoel. After 96 h post parasitization, PO activity and the percentage of melanization was slightly recovered, and it is just the time that the endoparasitoid larva had well developed in host and was able to avoid the host immune system. The recovery of host immune response would protect the parasitoid from other opportunistic parasites and pathogens.

PDVs are unique viruses that have been found only in the families of Braconidae and Ichneumonidae of Hymenopetra. These viruses are characterized by double-strand, superhelical DNA genomes that are heterogeneous in size. Polydnaviruses are widely studied on their immunosuppression to the host since the first were isolated from ichneumonid *Nemeritis canescens* by Rotheram[15]. They are required for successful development of the endoparasitic wasps. In some cases, host hemolymph phenoloxidase activity and melanization reaction were inhibited after parasitism[5,6,9]. In our study, both PO activity and melanization reaction in hemolymph of *H. armigera* were suppressed by parasitizing of *C. chlorideae* or injecting of its calyx fluid. So we speculated that the influence on PO activity by *C. chlorideae* was mainly due to the components of calyx fluid in this parasitoid. The calyx fluid of *C. chlorideae* contains ovarian proteins and plenty of polydnaviruses (unpublished observation). Ovarian proteins exert influence on host immunity only at the early stage of parasitism by *C. sonorensis* (befor 24 h)[16], while PDV gene must be expressed before it affected host immune response. The expression time and expression level of *C. chlorideae* PDV in *H. armigera* were consistent with the strong inhibition of hemolymph PO activity (unpublished data). So we suggested that inhibition of PO activity post parasitization or calyx fluid-injection was mainly caused by *C.chlorideae* PDV, and not by ovarian proteins.

The inhibition of PO activity and melanization was considered to relate to some enzyme activities during melanin formation. It is reported that the activities of dopachrome tautomerase and DOPA decarboxylase (DDC) are dramatically reduced by CsPDV infection, while PO, DDC and dopachrome tautomerase are responsible for the melanin formation from tyrosine[17]. After *C. sonorensis* calyx fluid was injected into *H. virescens* larvae, the concentration of substrates for melanin formation, tyrosine and tyrosine glucoside in host plasma was decreased[17]. The inhibition of hemolymph melanization by parasitism

was due to the reduction of PO activity, but the mechanism of PO activity suppression is still unknown. PO usually occurs in an inactive proenzyme form (proPO) in the hemolymph of most insects[2,10]. The activation of proPO cascade can be triggered by specific serine protease, so the inhibition of PO activity can occur at different levels, and the regulation mechanisms of PO activity by parasitoids may vary in different host-parasitoid systems. Recently, Beck et al.[18] reported that the calyx fluid of ichneumonid *Venturia canescens* has a potential ability to inhibit PO activity in host hemolymph due to the presence of a putative serine protease inhibitor activity. In our study, we first use the additional active bovine trypsin to assay the mechanism of PO inhibition. We proved that bovine trypsin could activate the proPO in unparasitized or Ringer's solution-injected haemocytes, whereas there was no activated activity of proPO in parasitized or calyx fluid-injected haemocytes. Bovine trypsin is a no-specific serine proteinase. Over degradation of proPO by high concentration of this enzyme may result in the reduction of the PO activity. In our assay, 5 mmol/L Ca^{2+} contained in the reaction mixture will also activate proPO[12]. During 60 min reaction, bovine protease could increase PO activity remarkably in unparasitized and Ringer's solution-injected sample (figs. 2 and 3), but could not in parasitized and calyx fluid-injected samples (figs. 4 and 5). We suggested that in the unparasitized hemolymph high proPO concentration existed, and/or could be completely activated by trypsin; in parasitized hemolymph the concentration of proPO was low, and/or would be over degraded by high concentration of bovine trypsin instead of being activated. The inhibition of *H. armigera* PO activity by *C.chlorideae* perhaps is due to the reduction of proPO expression or the decrease of proPO concentration by rapid degradation after parasitism.

Acknowledgements This work was supported by a special fund for the Major State Basic Research Project of China (Grant No. 2000016208) and the National Natural Science Foundation of China (Grant No. 30070517).

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(Received May 14, 2001)