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EFFECT OF ENHANCING FACTOR ON THE DEVELOPMENTAL RESISTANCE OF *PSEUDALETIA SEPARATA* LARVAE ORALLY INOCULATED WITH *PSEUDALETIA UNIPUNCTA* NUCLEOPOLYHEDROVIRUS

PENGARUH "ENHANCING FAKTOR" TERHADAP MEKANISME RESISTENSI LARVA PSEUDALETIA SEPARATA *YANG DIINOKULASI SECARA ORAL DENGAN NUKLEOPOLIHEDROVIRUS* PSEUDALETIA UNIPUNCTA

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INTISARI

Larva Lepidoptera akan semakin resisten terhadap infeksi Baculovirus seiring dengan pertambahan umur larva. Mekanisme resistensi ini belum secara jelas diketahui. Penambahan "enhancing factor" pada inokulum virus dapat mematahkan resistensi ini. Hasil penelitian ini menunjukkan bahwa mekanisme resistensi terjadi pada infeksi primer di usus tengah larva. Dengan mengukur fusi antara microvilli sel dengan virus menggunakan flow cytometer, diduga "enhancing factor" membantu virus untuk fusi dengan microvilli sel pada larva yang tua.

Kata kunci : *Enhancing factor, Baculovirus, resistensi*

ABSTRACT

Larvae of lepidopteran insect become increasingly resistant to baculovirus infection as they age. The mechanism for this resistance is not known yet, but the phenomenon does not occur when an enhancing factor was added to the viral inoculum. This observation indicated that the mechanism of resistance occur during primary infection within midgut. By assessing the fusion of positive microvillus cell using flow cytometer, we indicated that enhancing factor may somehow help the virus to fuse in the midgut of older insect

Key word : Enhancing factor, baculovirus, developmental resistance

INTRODUCTION

The genus *Nucleopolyhedrovirus* (family *Baculoviridae*) compromises arthropod-specific viruses that can produce fatal infection in their host, primarily larval lepidopterans. Baculoviruses are known to infect over 500 different species of insects, including such serious insect pest species such as the codling moth, cotton bollworm, cabbage looper, European pine sawfly, potato tuber worm, forest tent caterpillar, gypsy moth, tobacco budworm, beet armyworm, imported cabbage worm, and spruce budworm (Washburn *et al.*, 2001).

Although baculoviruses are becoming recognized as important agents for the control of insect pests, only a few of them have been produced in quantities sufficient for commercial use as insecticides. One important obstacle comes from developmental resistance, the phenomenon whereby host larvae become progressively more resistant to fatal infection as they age within and among instars (Washburn *et al.*, 2001). From practical standpoint, this phenomenon impacts the effectiveness of baculovirus control programs of agricultural and forest insect pests because it is necessary to adjust application levels in the response to the demography of the target insect population (Engelhard and Volkman, 1995). Very little is known about these events for a number of reasons, one of them being that oral inoculation of larvae with biologically relevant doses of virus results in infection of only a few midgut cells. Indirect evidence indicates that the mechanism of resistance may involve events that take place during infection of the midgut. (Engelhard *et al.*, 1994).

Xu and Hukuhara (1992, 1994) reported the isolation of a virus-enhancing factor (EF) that enhances nucleopolyhedrovirus (NPV) infection in the armyworm, *Pseudaletia separata*, from the spheroid of *P. separata* entomopoxvirus (PSEV). A possible application of the EF will be in the area of insect pest control by NPVs by using it as a synergist, thereby potentially reducing the amount of NPV needed for successful application to crops (Hukuhara and Wijonarko, 2001).

In this report, we quantified the mortality levels for four cohorts of fifth-instar *P. separata* with a constant dose of a *Pseudaletia unipuncta* MNPV. Each cohort differed in age by twelve and twenty-four hours, yet there were decreasing levels of mortality in successively older cohorts. We found that the presence of EF, increasing the infectivity of the NPV in the older cohort. We also purified the brush border membrane vesicle (BBMV) and analyze in flow cytometer to investigate the relationship between EF and the degree of virus fusion in older cohort.

MATERIALS AND METHODS

Insects. A colony of *P. separata* reared at 25°C was used in this experiment. The adults were maintained on 18L: 8D cycle in carton box and were provided with a sterile 10% sucrose solution. Larvae were reared with artificial diet according to Hattori and Atsuzawa, (1980). Some of these larvae were held at 8°C to adjust the timely development

Virus. The origin of *P. unipuncta NPV* and the methods of purifying the inclusion bodies were according to Hukuhara and Zhu, (1989). Severely infected larvae were macerated in distilled water within a mortar and tissue debris was excluded by differential centrifugation. Purified polyhedra were stored at --30°C before use.

Bioassay. Fifth and sixth-instar larvae of *P. separata* were used in this experiment. Bioassay was conducting according to Wijonarko and Hukuhara (1998) with little modification. Newly molted fifth-instar (5^0), newly molted sixth-instar (6^0), 12 hours old sixth-instar (6^{12}), and twenty-four hours old sixth-instar were used for bioassay (6^{24}).

Preparation of BBMV. BBMV were prepared with ultrasonication based on the method developed by Cioffi and Wolfersberger (1983) with some modifications as follows. 50 - 60 last stage of *Pseudaletia unipuncta* larvae were used for every preparation of BBMV. Larvae were immobilized by chilling in ice for at least 20 min. Larvae were excised longitudinally with dissecting scissor and the midgut was removed with forceps. Malpighian tubules, peritropic membrane, other unnecessary tissues, and remaining diet were also removed. Midguts were rinsed in buffer containing 0.25M sucrose, 5mM EDTA, 5 mM Tris, 0.1 mM PMSF (pH 8.1). They were then transfer into fresh medium and cut into small pieces, cooled on ice and treated with ultrasonicaton (Branson Sonifier 250) for 30 seconds on its lowest setting to create uniformly size vesicle. After filtering with gauze filter, the midgut suspensions were centrifuged at 4000*g* for 15 minutes using an angle rotor centrifuge (Hitachi, Himac CR 20, Japan). The supernatant was aspirated from the tube without disturbing the pellet, and re-centrifuged at 4000*g* for 15 min. The resulting supernatants were again centrifuged at 10,000*g* for 30 min. The pellet resulting from the final 10,000*g* centrifugation were resuspended in buffer containing 50 mM sucrose, 5 mM EDTA, 0.1 M CAPS, 0.1 mM PMSF (pH 10.8).

Preparation of EF. EF was prepared by solubilized the spindle of PsEV in dissolution buffer (0.6 M Na_2CO_3; 0.015 sodium thioglycolate; 0.03 M EDTA). An equal amount of distilled water was added to stop the reaction. Solubilized spindle protein were dialyzed overnight against 0.01 M Tris-HCl (pH 8) and applied onto an affinity column (Hi-Trap NHS-activated, Pharmacia Biotech., Uppsala, Sweden) that had been coupled with immunoglobin from an anti-EF rabbit anti serum. Active fraction were dialyzed overnight against phosphate-buffered (0.1 M PBS, pH 7.4) at 4°C, and stored at --30°C until used.

Statistic analysis. ID_{50}, slope (*b*) and their standard errors were calculated as described by Berkson (1955). The value of 95% fiducial limits were calculated from standard error according to Zar (1987).

RESULTS

Bioassay results showed that armyworm increased their virus resistance against the NPV as they get older. Larvae orally administered with 10^4 polyhedra/larva decreased their mortality rate as they age and even there was no NPV infection detected in the larva of 24 hrs old sixth instar larvae (Fig.1). In contrast, when the inocula were combined with the purified EF, there was no significant impact of insect age on larval mortality. The cumulative mortality of armyworm larvae administered with 10^4 polyhedra/larva in instar 5^0, 6^0, 6^{12}, and 6^{24} with the addition of purified EF were 100; 85; 85; and 85 percent, respectively. In the absence of EF, the mortality was 25; 15; 10; and 5 percent, respectively (Fig 1, A-D). Quantitative bioassay results showed that between larvae at the same age, there were significant differences in ID_{50} value, in the presence of EF compared to the larvae that received only polyhedra (Table 1). Without EF, the ID_{50} value of the 6^{24} instar larvae could not be define due to low level mortality. On the other hand, with the addition of EF there were no significant difference in ID_{50} value of sixth instar larva, which indicates that the EF may in someway help the initial infection within the armyworm midgut.

Fusion assay to explore further the relationship between BBMV and NPV infection was performed by purifying the BBMV from columnar cell, and was analyzed with flow cytometer EPICS ALTRA (Beckman Coulter™). The analysis result showed that in the presence of the EF positive cell after 30 min, 60 min, and 90 min mixing with PuNPV was 15 ; 27 and 45 percent, while in the absence of EF was 0; 4 and 12 percent (Fig. 2).

DISCUSSION

Bioassay results showed that in the presence of EF, the ID_{50} value of the fifth and sixth-instar larvae were low compared to those in the absence of EF. In the absence of EF the ID_{50} were high, and in the 24 hours old sixth instar larvae the resistance did occurred. Engelhard and Volkman (1995) showed that mature instar of Lepidopterans larvae have the

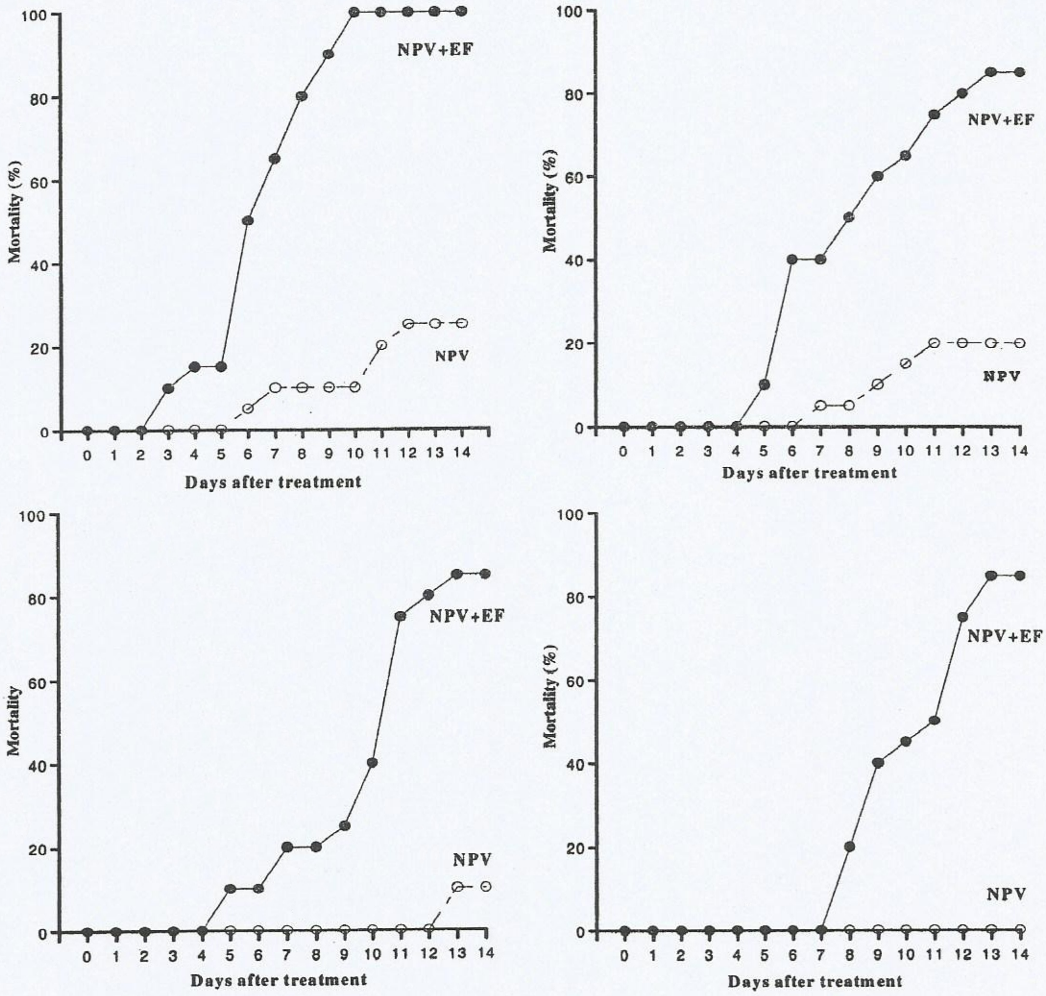

Figure 1. Cumulative mortality of: (A)newly molted fifth instar larvae; (B) newly molted sixth-instar larvae (C) twelve hours old sixth-instar larvae; (D) twenty four hours old sixth-instar larvae of armyworm administered with 10^4 polyhedra and purified EF 2.5 μg per larva. For each treatment 20-30 larvae were used

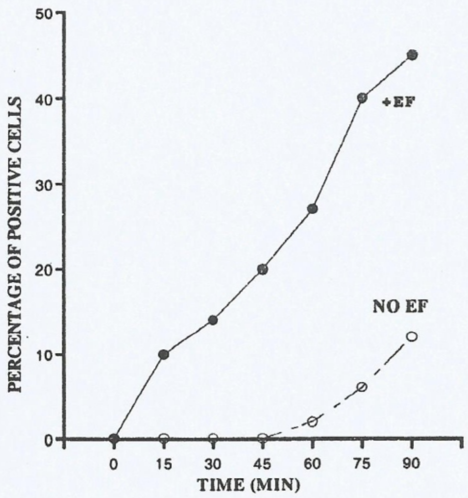

Figure 2. Kinetics of fusion of BBMV with polyhedra derived virions of PuNPV in the presence and in the absence of the EF.

Table 1. Comparison of the Infectivity of NPV in the Presence, and in the Absence, of the EF against Different Instar of *Pseudaletia separata* Larvae[a]

Instar	Slope (b)±SE	ID50 (polyhedra/larva)	95% Confidental Limit	$\chi^?$
PuNPV only				
5^0	0.70±0.21	1.08×10^5	4.61 – 5.45	2.82
6^0	0.68±0.15	1.14×10^6	5.49 – 6.63	0.45
6^{12}	0.60±0.16	3.35×10^6	5.81 – 7.25	4.69
6^{24}	*	*	*	*
PuNPV+EF				
5^0	0.57±0.14	3.69×10^0	-0.16 – 1.30	1.94
6^0	0.59±0.15	3.07×10^2	2.19 – 2.79	4.84
6^{12}	0.58±0.14	3.14×10^2	2.01 – 2.99	4.15
6^{24}	0.67±0.11	3.57×10^2	2.04 – 3.06	10.97

[a] Twenty larvae used for each dose. Each larva received 2.5 μg EF

* ID_{50} value could not be determined due to low level mortality

ability to avoid and to remove the primary foci of infection that lead to resistant against virus infection. Until recently, the mechanism on how the resistance happens is still unclear. Wang and Granados (1997) showed that peritropic membranes of the *T. ni* larvae contain an invertebrate intestinal mucin, which is target substrate for a baculovirus enhancin. On contrary, Washburn *et al.*, (1995) suggested that the presence of peritropic membrane did not impart significant protection from viral infection based on their observation that the PM was absent in newly-molted *T. ni* larvae, and that there was no significance difference in the mortality levels between larvae with and without peritropic membrane.

Our recent unpublished data which showed that intrahemocoelly injection with NPV in the older larvae resulted in the massive NPV infection, suggested that the EF may in someway break this defense mechanism in the midgut and helps the massive NPV infection. This hypothesis was also supported with the data from immunofluorescence analysis using confocal microscope which showed that the BBMV has specific affinity against EF, and the BBMV may play an important role during the primary infection of NPV in the midgut larvae (unpublished data). Flow cytometer analysis of purified BBMV cells showed that the positive number of cell were increasing as time goes, and that the presence of EF will greatly enhance the number of positive cells. It is tempting to speculate, that the EF may somehow enhance the infectivity of NPV and break the developmental resistance in the older stage of armyworm larvae.

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