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A Genetically Modified Nucleopolyhedrovirus: Effectiveness and *in vivo* Expression in the Black Cutworm, *Agrotis ipsilon* (Huf.) (Lepidoptera: Noctuidae)

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ABSTRACT

Baculoviruses are orally infectious to insects and are considered as viral insecticides and safe for the environment. To enhance their speed-of-kill, these viruses were genetically engineered to express insect enzymes. Efficiency of a recombinant virus, *Autographa californica* multiple nucleopolyhedrovirus expressing *Manduca sexta* juvenile hormone esterase (*AcMs*JHE), and its wild type (*Ac*MNPV) for killing 1st instar larvae of *Agrotis ipsilon* (Huf.) was analyzed. Mortality rate of *A. ipsilon* larvae was higher with recombinant virus than wild type by 1.4 times on the level of LC_{50}; at the same time it was 16.7% faster in killing than its wild type. Effect of virus infection on larval weight indicated that 3rd instar larvae infected with 1000 PIBs/larva were dramatically reduced in weight with either recombinant or wild type viruses, although the percentage of reduction was higher with recombinant than wild type virus. Isoelectric focusing analysis showed that there were expression of juvenile hormone esterase *in vivo*, and it is believed that it is related to gene expression. These results indicated that the genetically engineered baculovirus, *AcMs*JHE, could provide effective control compared with its wild type for *A. ipsilon* and further support the potential use of genetically engineered baculoviruses in insect pest control.

Key words: *Agrotis ipsilon*, Recombinant Nucleopolyhedroviruses, bioassay, JHE Expression.

INTRODUCTION

Baculoviruses are a group of arthropod-specific viruses that have been isolated mostly from larvae of Lepidoptera (Bonning, 2005). All baculoviruses belong to a single family, Baculoviridae, which is currently composed of the genera *Nucleopolyhedrovirus* and *Granulovirus* (Theilmann *et al.*, 2005). Baculoviruses have a narrow host range, are highly pathogenic and have occlusion bodies which make them more environmentally stable than some other families of entomopathogenic viruses (Entwistle and Evans, 1985). These characteristics enable baculoviruses to be used as microbial control agents against insect pests. There are many examples of successful use of baculoviruses in this role (Entwistle, 1998 and Moscardi, 1999).

The black cutworm, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae) is a worldwide pest. It lives on the ground, where it feeds on seedlings of nearly all vegetable and field crops, most important crops include tobacco, cotton, tomato, potato, cabbage, barley and oats (Rings *et al.*, 1975). All instars of *A. ipsilon* feed on the leaves of corn seedlings but the most serious damage results from leaf and stem cutting by late instars (Clement and McCartney, 1982). They are often detected only when the plants are already severely damaged. In the past, several species of entomopathogenic bacteria, protozoa, fungi and viruses were isolated and evaluated as possible biological control agents of cutworm pests (Lipa, 1971; Ignoffo and Garcia, 1979 and Cossentine and Lewis, 1986). Cutworms could be controlled with different baculoviruses isolated from diseased insects (Zethner *et al.*, 1987 and Caballero *et al.*, 1991a, b).

Baculoviruses have been considered as viral insecticides and are safe for the environment (Cheng and Lynn, 2009). Although several viral insecticides with advantageous characteristics have been registered, their use is still limited. A major disadvantage of viral insecticides is that the speed of insect killing is relatively slow, allowing infected pest larva to continue to cause economical damage to crops. Recombinant DNA technology used to eliminate the disadvantages of viral insecticides by contributing to the acute insecticidal effects induced by inserted or deletion genes in baculoviruses, several innovative and successful approaches have been taken to improve the speed of kill of baculoviruses through genetic modification (Kamita *et al.*, 2005). Most examples of GM viruses utilize viruses of the Baculoviridae family and include exogenous gene insertions such as those encoding insect specific toxins (Merryweather *et al.*, 1990; Maeda *et al.*, 1991; Gershburg *et al.*, 1998; Harrison and Bonning, 2000 and Regev *et al.*, 2003), hormones (Eldridge *et al.*, 1991, 1992a and b), neuropeptides (Ma *et al.*, 1998) and enzymes (Bonning *et al.*, 1992 & 2002; Gopalakrishnan *et al.*, 1993 and Harrison and Bonning, 2001).

In this study, the susceptibility of *A. ipsilon* to a recombinant nucleopolyhedrovirus (*Autographa*

californica Multi Nucleopolyhedrovirus carrying *Manduca sexta* Juvenile Hormone Esterase gene, *AcMs*JHE) was investigated as well the effect of the recombinant virus on larval weight was estimated.

MATERIALS AND METHODS

Insect and viruses

A laboratory colony of *A. ipsilon* was obtained from the Department of Cutworms, Plant Protection Research Institute, Agricultural Research Centre (ARC), Dokki, Giza, Egypt. The insect larvae were kept under laboratory conditions (27±3°C, 60–80% relative humidity and a 14:10h day: night photoperiod). The larvae were reared on a modified version of semi-synthetic bean diet (Gelernter *et al.*, 1986). The composition of the diet was as follows: 500 gm white beans; 150 gm brewer's yeast; 10 gm ascorbic acid; 5gm methyl-*p*-hydroxy benzoate; 2 mg sorbic acid; 30 gm agar; 10 ml formalin (removed in bioassay experiments) and 1200 ml distilled water. *AcMs*JHE (a recombinant baculovirus carrying the wild-type *Manduca sexta* Juvenile hormone esterase (MsJHE) (Kamita *et al.*, 2003), or AcMNPV (a wild-type baculovirus, *Autographa californica* multi Nucleopolyhedrovirus) were kindly provided by Dr. Bruce D. Hammock, Pesticide Biotechnology Laboratory, Department of Entomology and Cancer Research Center, California University at Davis, CA, USA.

Propagation and semi-purification of virus occlusion bodies (OBs)

Black cutworm larvae were reared individually in 1-oz. cups on the diet under the laboratory conditions. When 3rd instar larvae were preparing to molt (as evident from head capsule slippage), diet was removed from the cups and the larvae were starved for at least 6 hrs. Larvae that had molted to 4th instar were fed an 8 mm^3 cube of diet, on which 5.75 x 10^4 *Ac*MsJHE or *Ac*MNPV polyhedra had been pipetted for 48 hrs and then transferred on fresh diet.

OBs were isolated from larval cadavers as described by El-Sheikh (2006). Briefly, infected larvae were homogenized in distilled water and the suspensions were filtered through cheesecloth. The homogenate was centrifuged at 1000 rpm for 15 minutes. The pellet was re-suspended in 0.5% sodium dodecyl sulfate (SDS) and 0.1% sodium deoxycholate and incubated at 37°C for 2 hours. The suspension was refiltered through two layers of cheesecloth and centrifuged at 1000 rpm for 15 minutes. The pellet was re-suspended in 30 ml of distilled water and centrifuged at 1000 rpm. This process was repeated three times, and the pellet was re-suspended in sterile distilled water to give a final concentration of approximately $8.5x10^8$ PIBs/ml which were counted using a counting chamber (Brightline Hemocytometer) according to Martin (1990). The semi-purified OBs were stored at -20 °C until used.

Bioassay and time-mortality determination

First instar larvae were used for the study within 6 hrs of egg hatching and lethal concentration bioassays were carried out using a droplet feeding technique (Hughes and Wood, 1981). Larvae were allowed to drink from virus suspensions of distilled water containing 10% w/v sucrose and 5% w/v blue food coloring dye. Five concentrations ranged from 100 to 2000 PIBs/µl were applied in droplets (1 µl each) in 60 mm disposable Petri-dishes, in which 35 to 40 larvae were exposed to each concentration for 15 minutes, then transferred individually into bioassay trays (1 larva per well) containing fresh diet using paint brushes. The time of transfer was noted as time zero and mortality was determined at 48 hr. intervals. Larvae that died during the initial 12 hr. period were considered injured by the treatment and discarded. The experiments were stopped at 10 days post inoculation (p.i.) or earlier, if all of the larvae had died. Bioassays were replicated at least twice.

Time-mortality tests were conducted using 1000 PIBs/µl concentration of each virus. The droplet feeding bioassay technique was conducted as mentioned before, and 30 individuals, 1st instar larvae were tested for each virus. In the control treatment, droplets of distilled water were used. The larvae were examined for mortality every 12 hours, beginning 12 hours post infection until they died or pupated. Larvae died or seriously injured before 12 hours post infection were considered to have been damaged during transfer. Median lethal time (LT_{50}) values were calculated. Probit analysis (Finney, 1971) was performed for lethal concentration and lethal time calculations using the MLP software package.

Effect on larval-weight

For analysis of infection's effect on larval weight, 3rd instar larvae of *A. ipsilon* were fed on a virus dose of 1000 PIBs in 1 µl droplet of *AcMs*JHE or *Ac*MNPV viruses, as described previously. Larvae that consumed the dose (1 µl droplet containing 1000 PIBs) within 10 min. were used. Control larvae were fed only on distilled water. The experiments were performed with 10-15 larvae per treatment. Following inoculation, the larvae were transferred individually into 1-oz cups containing fresh diet. After 5 days, the larvae were weighted.

Total haemolymph protein determination and IEF analysis

For total haemolymph protein determination and *in vivo* JHE expression, larvae inoculated similarly as in the previous experiment (infection's effect on the larval mass), and then three to four infected ones were bled at 24 hours post-infection (h.p.i.) or 5 days post-infection (d.p.i.). Haemolymph samples were stored with a crystal of phenylthiourea at -20°C prior to Bio-Rad protein assay (Bio-Rad Laboratories, 1977) using bovine serum albumin (Fraction V; Fisher Scientific, Pittsburgh, PA) as a standard or IEF separation for JHE analysis. The infected larvae were reared at 26°C, 60% R.H. and 12:12h light: dark cycle.

Isoelectric focusing (IEF) was performed with Novex pH 3-10 IEF (Invitrogen) gels, and running buffer recommended by the manufacturer. Following IEF gel electrophoresis, esterase activity was detected by naphthyl acetate staining by soaking the gel in 50 mM sodium phosphate buffer, pH 6.8, containing 0.05% Fast Blue RR salt (Sigma-Aldrich), 0.02% α-naphthyl acetate (Sigma-Aldrich), and 0.02% β-naphthyl acetate (Sigma-Aldrich) at 35°C.

Statistical analysis

SPSS 10.0 for Windows software package was used for statistical analyses. One-way ANOVA analysis of variance was performed and variant groups were determined by means of the LSD method to compare differences among treatments at the $p<0.05$ (bioassays and time-mortality) or $p<0.01$ (reduction in larval weight).

RESULTS AND DISCUSSION

Bioassay and time-mortality response

Comparative infection experiments using neonate larvae of *A. ipsilon* were carried out to determine the virulence of *AcMs*JHE and *Ac*MNPV. Mortalities from virus infection of the larvae were scored 10 days post-infection and the LC_{50} values were calculated from the observed mortalities corrected against the mortality of mock larvae using the formula of Abbott (1925). The LC_{50} values were 396 and 570 PIBs/µl of *AcMs*JHE and *Ac*MNPV, respectively (Table 1). The efficiency of *AcMs*JHE in causing mortality for *A. ipsilon* neonate larvae was 1.4 times more than *Ac*MNPV.

Table (2) shows the time mortality response of neonate larvae of *A. ipsilon* that infected at a concentration of 1000 PIBs/µl. The LT_{50} values indicate that the recombinant virus, *AcMs*JHE, was 16.7% more effective and faster compared to *Ac*MNPV, where the time required for 50% kill were 140 and 168 hr. with *AcMs*JHE and *Ac*MNPV, respectively.

In order to analyze the susceptibility of *A. ipsilon* to a recombinant nucleopolyhedrovirus (*AcMs*JHE), bioassay experiments were conducted to compare the recombinant virus with the wild type on 1st instar larvae. Data indicated that the recombinant virus was higher in efficiency by 1.4 times than the wild type one. At the same time, the determination of time-mortality was an important and complementary measurement with bioassay to figure out the efficiency of viruses. Also, the recombinant virus exhibited 16.7% higher in speed of killing 1st instar of *A. ipsilon* larvae compared with its wild type, giving other evidence about the efficiency of recombinant over wild type virus. Several studies were carried out to investigate the effectiveness of recombinant viruses on many lepidopteran insects and showed the efficiency of recombinant against wild types viruses (Merryweather *et al.*, 1990; Eldridge *et al.*, 1991, 1992a, b; Maeda *et al.*, 1991; Bonning *et al.*, 1992

Table (1): Median lethal concentrations (LC_{50}s) of *AcMs*JHE and *Ac*MNPV in bioassays on neonate larvae of *A. ipsilon*

Virus	n*	LC_{50} (95% CL)[a]	Slope ± SE[b]	Heterogeneity (X^2/df)	Relative Potency[c]
*AcMs*JHE	225	396 (278-523)	1.9±0.3[a]	1.9	1.4
*Ac*MNPV	228	570 (390-767)	1.8±0.3[a]	2.3	-

* n refers to the total number of larvae tested/virus.

[a] LC_{50} and 95% confidential limits (CL) are given in number of occlusion bodies/ml diet.

[b] Values indicate that probit lines are parallel ($P<0.05$), and followed by the same letter.

[c] Relative potency of recombinant virus to wild type is calculated as LC_{50} of *Ac*MPNV/ *AcMs*JHE.

Table (2): Time-mortality response of *A. ipsilon* larvae infected with *AcMs*JHE and *Ac*MNPV*

Virus	n**	LT_{50} (95% CL)[a]	Slope± SE[b]	Heterogeneity (X^2/df)
*AcMs*JHE	30	140 (124–155)	4.4±0.5[a]	5.9
*Ac*MNPV	30	168 (152–184)	4.5±0.6[a]	4.1

* All larvae used in time mortality experiments were fed on a concentration equal 1000 PIBs for 15 minutes from each virus.

** n total larvae examined/treatment.

[a] LT_{50} and 95% confidential limits (CL) are given in hours post infection (h.p.i.).

[b] Values indicate that probit lines are parallel ($P<0.05$), and followed by the same letter.

Table (3): The effect of virus infection on larval weight of *A. ipsilon*

Treatments	N*	Mean± STD (mg)	95% Confidence Interval	
			Lower	Upper
*AcMs*JHE	12	28.00±3.21**	25.32	30.68
*Ac*MNPV	14	32.75±3.00**	30.27	35.23
control	10	42.13±4.26	38.57	45.68

*n is total number of 3rd instar larvae inoculated with 1000 PIBs/larva.

**Differences compared with control are significant at the $P<0.01$ level.

& 2002; Gopalakrishnan *et al.*, 1993; Gershburg *et al.*, 1998; Harrison and Bonning, 2000 & 2001; Regev *et al.*, 2003; Kamita *et al.*, 2005; El-Sheikh 2006; Inceoglu *et al.*, 2006 and Ashour *et al.*, 2007).

Larval-weight determination

Third larval instar of *A. ipsilon* infected with 1000 PIBs/larva of either *AcMs*JHE or *Ac*MNPV was used to determine the efficiency of both viruses in reducing larval weight. Results clearly indicated that larvae infected with *AcMs*JHE were 33.5% less in weight than control, while larvae infected with *Ac*MNPV were 22.3% less in weight than control (data was significant with P<0.01) (Table 3).

Larval weight determination gave an overview of the correlation between changes in the insecticidal efficacy determined on insect pest neonates and the actual *in vivo* effect of the engineered baculovirus. Since the effect of the recombinant virus was higher by 33.5% over control in reducing larval weight, this means that it prevented the inoculated larvae from consuming the same amount of food compared with un-inoculated larvae, meaning damage reduction (Regev *et al.*, 2006 and El-Sheikh *et al.*, unpublished data).

Total haemolymph protein and *in vivo* expression of JHE

Total haemolymph protein determined spectrophotometrically (Fig. 1) shows the decrease in haemolymph protein amounts in all treatments after 5 d.p.i. compared with control. The decrease was higher in the larvae treated with *Ac*MNPV than those treated with *AcMs*JHE.

Isoelectric focusing was used to analyze *in vivo* expression of JHE, where the haemolymph samples were taken after moulting. Wide range IEF gel (3–10) was used to determine isoelectric point (p*I*) of the JHE existing in *A. ipsilon* haemolymph. Fig. (2)

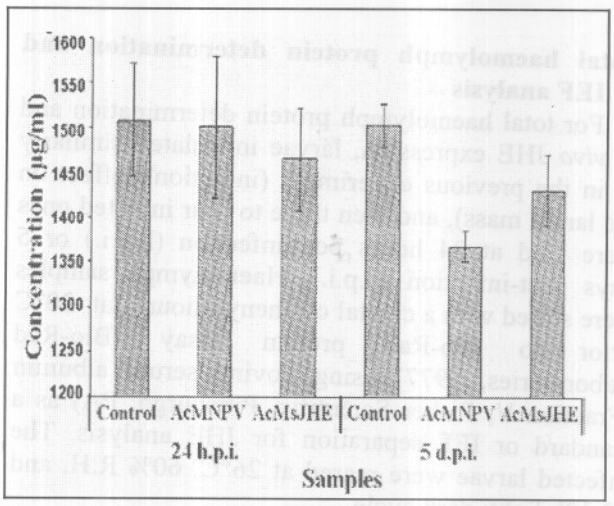

Fig. (1): Total haemolymph protein of *A. ipsilon* larvae treated with *AcMs*JHE and *Ac*MNPV, samples taken 24 hour and 5 days post infection.

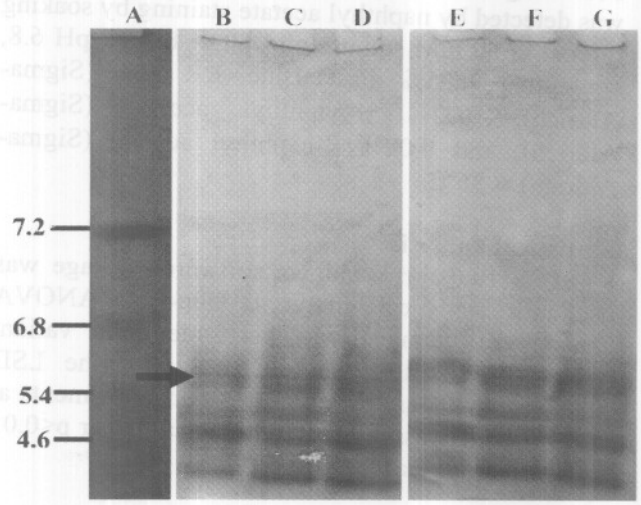

Fig. (2): IEF analysis of crude haemolymph for JHE expression (arrow). Samples were run on a Novex precast isoelectric focusing gel, wide range (pH 3–10). The resultant gel was stained with α & β-naphthyl acetate. IEF markers purchased individually from Sigma-Aldrich were run in lane A and stained with commassie brilliant blue. The p*I* of each marker is indicated next to the respective band. Lanes from B to G contain 15 µl of crude haemolymph. Samples loaded in lanes B-D were taken 24 hour post infection, while samples loaded in lanes E-G were taken 5 days post infection. Lanes (B&E) haemolymph samples from un-inoculated larvae, lanes (C&F) haemolymph samples from larvae inoculated with *Ac*MNPV, and lanes (D&G) haemolymph samples from larvae inoculated with *AcMs*JHE. Arrows indicate the appearance of JHE of approximately 5.6 p*I*.

shows that the level of JHE in samples taken 24 h.p.i. was low compared with those taken after 5 d.p.i., and JHE estimated to be 5.7 *PI*. At the same time, the JHE band in lane G (*AcMs*JHE treatment) was more intensive than that in lane F (*Ac*MNPV treatment).

JHE is a selective enzyme that hydrolyzes the methyl ester of juvenile hormone. This enzyme plays an important role in the regulation of metamorphosis in caterpillars, and is implicated in additional roles in development and reproduction in many insect orders. JHE protein from *Manduca sexta* (Hinton and Hammock 2001) and from *Tenebrio molitor* (Hinton and Hammock 2003a) were purified and cloned, then the full length coding region of the JHE cDNA from *M. sexta* was subcloned into the baculovirus *Ac*MNPV genome under the control of the p10 promoter (Hinton and Hammock, 2003b). The recombinant virus with *Ms*JHE demonstrated the expression of high levels of JHE activity when infecting Hi5 cells from *Trichoplusia ni*. Total haemolymph protein and IEF analysis of JHE from *A. ipsilon* hemolymph were investigated to know if there is gene expression of JHE protein in infected *A. ipsilon* larvae. The hemolymph protein analyzed calorimetrically showed that the amount of total protein in larvae that passed 5 days infection with *AcMs*JHE or *Ac*MNPV was low compared with the control, while in the larvae infected with *AcMs*JHE, it was slightly high compared with those infected with *Ac*MNPV. In comparison with JHE analyzed by IEF electrophoresis, the situation was different than total protein data. Fig. (2) shows that the band of JHE in haemolymph taken from larvae after 5 days of infection was intensive and big in size compared with larvae infected with wild type virus or uninfected. This means that the gene seems to be expressed inside infected larvae, giving evidence of the ability of genes to express in different hosts. Hinton and Hammock (2003b) stated that the JHE protein partially purified from Hi5 cells after infection with *AcMs*JHE by anion exchange chromatography, expressed similar features to the wild type protein extracted from *M. sexta*, and its MW estimated to be 66,500 Da.

In conclusion, *A. ipsilon* larvae seemed to be more susceptible for infection with *AcMs*JHE than *Ac*MNPV, meaning higher efficiency of the recombinant virus over the wild type on the level of LC_{50} or LT_{50}. Existence of an intensive band of JHE in haemolymph samples of larvae infected with recombinant virus was likely to be gene expression and provided new measures of possibility for using recombinant viruses with JHE gene insertion for *A. ipsilon* control.

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