Further enhancement of baculovirus insecticidal efficacy with scorpion toxins that interact cooperatively

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Abstract We have studied whether the cooperative insecticidal effect of certain scorpion toxin pairs, namely either a combination of excitatory and depressant, or alpha and depressant scorpion toxins, would improve the efficacy of *Autographa californica* nucleopolyhedrovirus (AcMNPV) over a virus expressing only a single toxin, towards *Heliothis virescens*, *Helicoverpa armigera*, and *Spodoptera littoralis* larvae. The best result was achieved by combined expression of the excitatory toxin, LqhIT1, and the depressant toxin, LqhIT2, that provided an ET_{50} value of 46.9 h on *H. virescens* neonates, an improvement of 40% over the efficacy of wild-type AcMNPV, and of 18% and 22% over baculoviruses that express each of the toxins independently. These results demonstrate that significant improvement in efficacy of recombinant baculoviruses is obtainable with toxins that exhibit a cooperative effect, and may contribute to employ baculoviruses to replace hazardous chemicals in insect control.

Key words: *Heliothis virescens*; *Helicoverpa armigera*; *Spodoptera littoralis*; Recombinant baculovirus; Cooperative anti-insect scorpion toxin

1. Introduction

Baculoviruses are appealing means for selective, non-polluting control of Lepidopteran pests [1]. However, a major impediment in their agricultural use is the slow 'speed of kill' of infected larvae [2]. Attempts to improve the relative effectiveness of baculoviruses included expression of scorpion, mite, and spider neurotoxins under control of potent viral promoters (e.g. polyhedrin and p10) yielded a 30–45% reduction in effective time to paralysis (ET_{50}) [3–11]. Further increase in efficacy was achieved by utilizing various promoters for expression of *tox34*, a neurotoxin of the predatory mite *Pyemotes tritici*, that resulted in a 50–60% reduction in the ET_{50} to *Trichoplusia ni* and *Spodoptera frugiperda* larvae [8] or by inactivation of the viral ecdysteroid UDP-glucosyltransferase gene (*egt*) in combination with *tox*34 expression in *Helicoverpa zea* nucleopolyhedrovirus, which provided 46% reduction in ET_{50} values to *H. zea* neonates [12]. Thus, insecticidal efficacy could be improved, but it still required further amelioration towards the standards set by chemical insecticides such as pyrethroids [13]. It is hypothesized that by harnessing strong promoters capable of expression at an early stage of viral infection, or toxins with higher activity, further increase in insecticidal activity is attainable. Also, the metabolic fate of the toxin in the larval hemocoel could determine its insecticidal efficiency, thereby explaining differences in bioactivity of baculoviruses expressing either excitatory or depressant toxins [11]. Moreover, the prominent lethal effect exerted by relatively small amounts of toxin produced in the larva compared to injected toxins, implied that the toxins were most likely mediated to their target sites along with baculovirus propagation [11]. Based on these observations and cooperative effects demonstrated with specific scorpion toxin pairs upon injection [14], we have tested whether baculovirus-mediated expression of toxin pairs at the vicinity of their targets would further improve the insecticidal efficacy of recombinant *Autographa californica* nucleopolyhedrovirus (AcMNPV). Our results indicate unequivocally that co-expression of excitatory and depressant toxins by AcMNPV shorten significantly the time to paralysis of infected *Helicoverpa virescens*, *Helicoverpa armigera*, and *Spodoptera littoralis* larvae.

2. Materials and methods

2.1. Cell lines and viruses

S. frugiperda SF9 and *T. ni* BTI-TN-5B1-4 (BTI) cells were maintained and propagated in TNM-FH medium supplemented with 10% heat-inactivated fetal bovine serum [6]. Infection of the cells with wild-type AcMNPV (strain E2) and recombinant viruses, and plaque titration of virus stocks were performed as was described [6].

2.2. Construction of recombinant viruses

Five recombinant viruses were employed for expression of three distinct toxins (LqhαIT, [15]; LqhIT2, [16]; LqhIT1, [11]) in various combinations (Fig. 1). The constructs, bearing each a single toxin gene, were described previously [6,11]. The depressant, LqhIT2, and excitatory, LqhIT1 toxins cDNAs were cloned in opposite orientation in plasmid pAcUW51P2 [11] under the control of the polyhedrin promoter and transcriptional terminator, and the p10 promoter with the SV40 terminator, respectively, by utilizing existing *Bam*HI and *Bgl*II restriction sites (Fig. 1). The LqhαIT-cDNA under the control of the AcMNPV polyhedrin promoter [6], was inserted upstream to

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Abbreviations: AcMNPV, *Autographa californica* nucleopolyhedrovirus; PIBS, polyhedral inclusion bodies; ET_{50}, effective time to paralysis

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the resident polyhedrin promoter utilizing an existing *Mlu*I restriction site (Fig. 1). Plasmids were verified by DNA sequencing. Polyhedra-positive recombinant viruses, vAcLqIT1-IT2 and vAcLqαIT-IT2 were isolated from SF9 cells co-transfected with the transfer vectors and linearized polyhedra-negative AcMNPV DNA as described [17]., and subjected to three rounds of plaque purification.

2.3. Toxin expression

BTI cells were infected at a multiplicity of infection (m.o.i.) of 20 as described before [11]. Toxin production was detected immunochemically using rabbit anti-LqhIT1, anti-LqhIT2, and anti-LqhαIT sera that do not cross-react [6,11].

2.4. Biological assays

Oral infection of *H. virescens* and *H. armigera* neonates (less than 12 h old), was performed with polyhedral inclusion bodies (PIBs). Due to difference in their susceptibility to AcMNPV, *H. virescens* and *H. armigera* were tested by the droplet feeding and the diet contamination methods, respectively [6,10,11]. Forty-eight *H. virescens* neonates were fed for 30 min with 3×10^3 PIBs/μl in 5% sucrose and 1 mg/ml FD and C Blue 1 (Hilton Davis, Cincinnati, OH), triplicates. The various viral constructs were examined simultaneously. Larvae that had ingested the virus ('blue larvae') were transferred individually onto fresh diet. Paralysis or death of infected-larvae was monitored at 4–6 h intervals for the first four days and 10–12 h thereafter. For two-viruses-applications, the mixture contained equal amounts of PIBs of each virus and the total PIBs amount was identical to the single-virus application.

H. armigera neonates larvae were exposed for 24 h to 7.5×10^3 PIBs/mm^2 (to obtain ~95–100% mortality within seven days at 30°C, [4]) and tested as described for *H. virescens*.

ET_{50} values of paralysis or death [9] were determined using ViStat [18]. The resulting values for each virus were averaged and analyzed by one-way ANOVA, the means were separated by the LSD test [19].

Intrahemocoelic infection of 3rd instar *H. armigera* and *S. littoralis* larvae was performed by injection of budded baculoviruses (10^4 p.f.u./μl, 5 μl per larva; 18 larvae per sample, concentration that provides ~95–100% mortality within 7 days at 30 °C, triplicates). The analysis was performed as described above.

3. Results

3.1. Co-infection with baculoviruses expressing single toxins

The insecticidal effect on *H. virescens* larvae, obtained upon simultaneous infection with equal doses of viruses expressing LqhIT1, LqhIT2 and LqhαIT, was compared to the effect produced by infection of each virus alone. The virus pair, vAcLqIT1 and vAcLqIT2 (Fig. 1), reduced the median time to paralysis by 15% over vAcLqIT1 (ET_{50} = 48.8 h versus 57.3 h) and 18.7% over vAcLqIT2 (ET_{50} = 48.8 h versus 60.0 h) (Table 1). The effect produced by the virus pair, vAcLqαIT with vAcLqIT2, was similar to the effect of vAcLqαIT but improved 22.7% over vAcLqIT2 (Table 1).

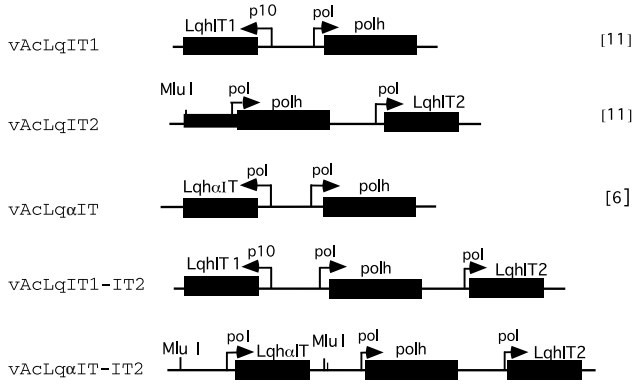

Fig. 1. Gene organization at the polyhedrin locus of recombinant baculoviruses bearing one or two toxin genes. The LqhIT1, LqhIT2 and LqhαIT genes were cloned in AcMNPV under the control of the p10 and polyhedrin (pol) promoters. Transcriptional start site is indicated by arrows. Polh: polyhedrin gene. vAcLqIT1, vAcLqIT2 and vAcLqαIT were previously assigned AcLIT1.p10, AcLIT2.pol [11] and AcLα22 [6], respectively. vAcLqIT1-IT2 and vAcLqαIT-IT2 bear the LqhIT1 and LqhIT2, or LqhαIT and LqhIT2, genes respectively.

Table 1
Time-response of *H. virescens* larvae infected per os with a baculovirus expressing synergistic anti-insect toxins

Virus	ET_{50}[a] ± S.E.M.	Slope ± S.E.M.
AcMNPV	79.1 ± 2.3 a	9.3 ± 1.3
vAcLqIT1	57.3 ± 1.9 b	9.9 ± 1.6
vAcLqIT2	60.0 ± 2.3 b	9.0 ± 1.6
vAcLqαIT	47.1 ± 1.3 c	12.2 ± 2.3
vAcLqIT1/vAcLqIT2[b]	48.8 ± 1.3 bc	11.1 ± 1.7
vAcLqαIT/vAcLqIT2[b]	46.4 ± 1.3 c	11.9 ± 2.0
vAcLqIT1-IT2	46.9 ± 1.8 c	8.3 ± 1.4
vAcLqαIT-IT2	45.6 ± 1.7 c	9.4 ± 2.1
F	10.54	0.70
P	< 0.01	> 0.6

[a]Effective time (hours post infection) to paralysis or kill 50% of *H. virescens* neonate larvae (less than 12 h old) infected at a LC_{95} dose of PIBs delivered by the droplet feeding assay. ET_{50}, standard error and slope were calculated using the ViStat program [18]. Small case letters indicate significant differences between treatments within the column.
[b]The mixture contained an equal amount of PIBs of each virus, the total amount of PIBs applied was equal to that applied with a single-virus.

3.2. Co-expression of toxins by a single baculovirus

In order to achieve concomitant expression of the recombinant toxins we constructed two baculoviruses each expressing a toxin pair: expression of LqhIT1 and LqhIT2 by vAcLqIT1-IT2 would be under the control of the very-late promoters, p10 and polyhedrin, respectively (Fig. 1), and expression of LqhαIT and LqhIT2 by vAcLqα-IT2 would be under the control of the polyhedrin promoter (Fig. 1). Infection of BTI cells with vAcLqIT1-IT2 or vAcLqα-IT2 resulted in comparable expression of all toxins that was also similar to the levels obtained upon expression of each toxin independently. (Fig. 2A,B)

Oral infection of *H. virescens* larvae with vAcLqIT1-IT2 provided ET_{50} values of 46.9 h versus 57.3 h and 60.0 h for vAcLqIT1 and vAcLqIT2, respectively (Table 1). However, infection with vAcLqαIT-T2 provided ET_{50} values of 45.6 h compared to 60.0 h and 47.1 h, respectively, for vAcLqIT2 and vAcLqαIT expressing a single toxin (Table 1).

Table 2
Time-response of *H. armigera* larvae infected per os with baculoviruses expressing synergistic toxins

Virus	ET_{50}[a] ± S.E.M.	Slope ± S.E.M.
AcMNPV	90.8 ± 3.1 a	9.1 ± 1.3
vAcLqIT1	71.0 ± 3.0 b	7.2 ± 1.2
vAcLqIT2	75.5 ± 3.4 b	7.2 ± 1.2
vAcLqαIT	66.4 ± 2.2 b	9.7 ± 1.6
vAcLqIT1-IT2	69.1 ± 2.4 b	8.1 ± 1.3
vAcLqαIT-IT2	64.3 ± 2.1 b	9.1 ± 1.4
F	6.58	2.62
P	< 0.01	> 0.05

[a]Effective time (hours post infection) to paralysis or kill 50% of *H. armigera* neonate larvae infected at a LC_{95} dose of PIBs delivered by diet incorporation. Small case letters indicate significant differences between treatments within the column.

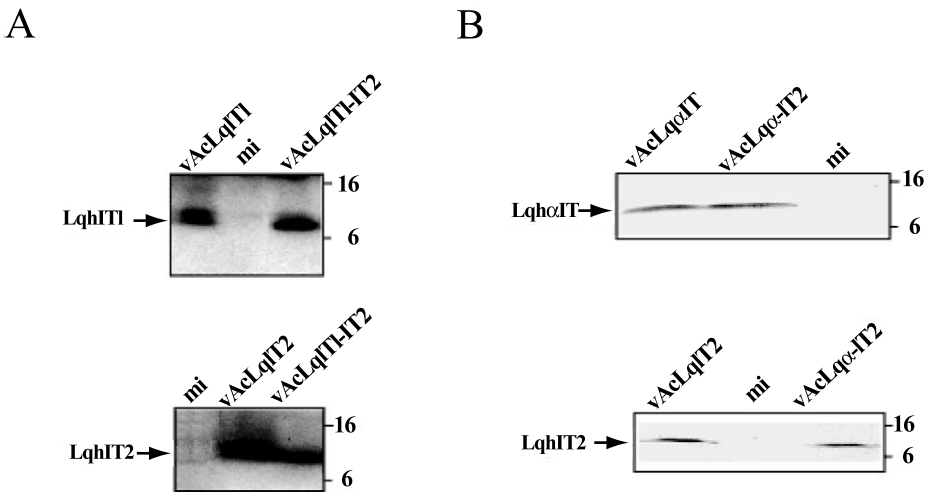

Fig. 2. Expression of toxins in insect cells. BTI-TN-5B1-4 cells were infected with: A, vAcLqIT1, vAcLqIT2 and vAcLqIT1-IT2, respectively; B, vAcLqαIT, vAcLqIT2 and vAcLqαIT-IT2 (vAcLqα-IT2), respectively. Cell extracts at 48 h post-infection were subjected to SDS–PAGE and immunoblot analysis using anti-LqhIT1 (A, upper panel), anti-LqhIT2 (A,B, lower panel) and anti-LqhαIT (B, upper panel) antisera. mi, mock-infected cell extracts. Arrows, indicate the corresponding toxins [6,11].

To validate the above results, we further infected other Lepidopteran species with our recombinant viruses: *H. armigera* (orally and via intrahemocoelic injection) and *S. littoralis* larvae (via intrahemocoelic injection). ET_{50} values obtained after oral infection of *H. armigera* with vAcLqIT1-IT2 or vAcLqαIT-IT2 were not significantly different from those achieved with the recombinants expressing the corresponding single toxin (Table 2).

Since *H. armigera* is semi-permissive to AcMNPV-infection [20], the anticipated cooperative effect of toxin pairs could be diminished by the non-efficient oral infection of the larvae. We tested this hypothesis by direct injection of budded viruses into the hemocoel of *H. armigera* larvae. Indeed, vAcLqIT1-IT2 was more efficient compared to the recombinant baculoviruses expressing each of the toxins separately, e.g. about 50% of the larval population infected with this recombinant was paralyzed within 72 h compared to 85 and 142 h of the larvae infected with vAcLqIT1 and vAcLqIT2, respectively (Fig. 3). vAcLqαIT-IT2 provided values of 50% paralysis at 64 h slightly better than vAcLqαIT (67 h) and much better than vAcLqIT2 (142 h) (Fig. 3).

We examined the various constructs on *S. littoralis*, which is non-permissive to AcMNPV oral infection [6]. Budded viruses were injected into the hemocoel of *S. littoralis* larvae and the estimated ET_{50} values obtained were approximately 54 h for vAcLqIT1-IT2 expressing toxin pairs, compared to approximately 97 and 74 h for viruses expressing single toxins, vAcLqIT1 and vAcLqIT2, respectively (Fig. 4). vAcLqαIT-IT2 provided values of 50% paralysis at 61 h better than vAcLqIT2 (74 h) and much better than vAcLqαIT (100 h).

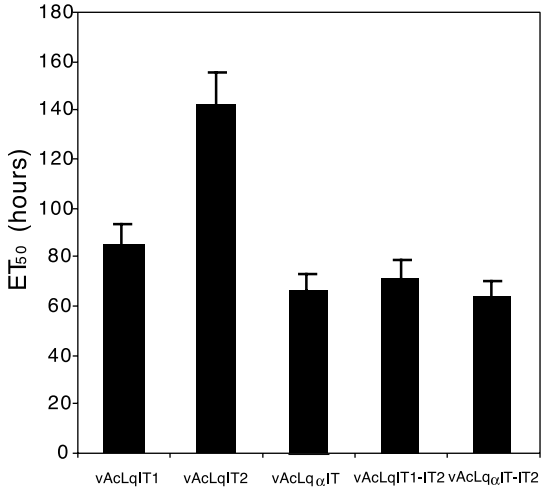

Fig. 3. Effect of budded virus injection to *H. armigera* larvae. 5×10^4 budded viruses were injected to 3rd instar larvae (18 larvae per tested virus, in triplicates). ET_{50} values were determined as the time post infection in which 50% of the tested larvae were paralyzed or dead.

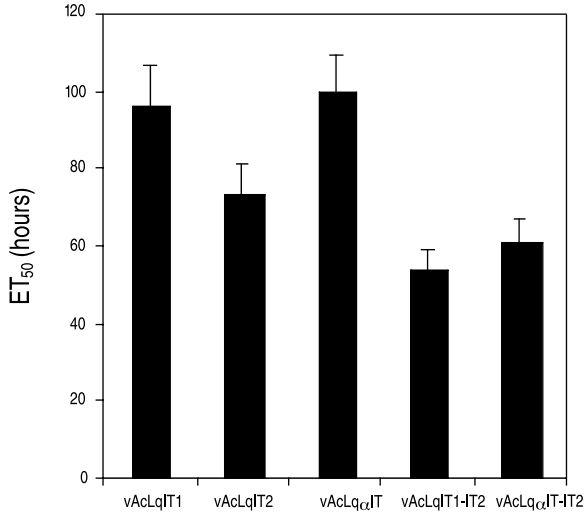

Fig. 4. Effect of budded virus injection to *S. littoralis* larvae. 5×10^4 budded viruses were injected to 3rd instar larvae (18 larvae per virus tested, in triplicates). ET_{50} values were determined as the time post infection in which 50% of the tested larvae were paralyzed or dead.

4. Discussion

Major increase in agricultural productivity has resulted from pest control by chemical insecticides directed at neuronal sodium channels. However, this approach is threatened by resistance build-up among insects and poisoning of non-targeted organisms (due to sodium channel mutations or evolutionary conservation of these channels, respectively). One solution to these problems envisions naturally occurring insecticidal viruses that can also serve as platforms for mobilization of anti-insect selective toxins. Baculoviruses that expressed arthropod borne anti-insect toxins has been shown to significantly reduce the time required to kill or paralyze 50% (ET_{50}) of the tested insect population. Moreover, risk assessment studies on non-target organisms have indicated that their future utilization could be environmentally safe [13,21].

Since scorpion excitatory, depressant, and alpha toxins bind to distinct receptor sites on the sodium channel [22], their combined application is expected to increase intoxication of the injected animal. This hypothesis was verified in toxicity assays using blowfly and *H. virescens* larvae resulting in a 10- and 5-fold improvement in effect over each of the toxins alone [14]. Yet, it remained to be elucidated whether such improved toxic effect would also be obtained when the toxins were mediated to their target sites by baculoviruses. As we show, co-production of LqhIT1 and LqhIT2 in *H. virescens* larvae driven by AcMNPV very late promoters, reduced the ET_{50} by 18–22% compared to larvae infected with recombinant viruses that express only one of the toxins (Table 1). This effect was less pronounced in orally infected *H. armigera* larvae (Table 2) probably due to differences in the metabolic fate of the two toxins in *H. virescens* and in *H. armigera*, or perhaps due to the lower susceptibility of *H. armigera* to AcMNPV infection [20]. *H. zea* larvae, which are non-permissive to AcMNPV infection, halt the virus spread by encapsulating the virions [23] but immunosuppression of the larvae enabled AcMNPV to infect them systemically. Thus, it was possible that the additional effect produced by scorpion toxin pairs was masked by the *H. armigera* immune response. Therefore, we analyzed whether such additional effect could be obtained in the semi-permissive *H. armigera* and the non-permissive *S. littoralis* larvae by direct intrahemocoelic injection of recombinant vAcLqIT1-IT2 and vAcLqαIT-IT2 budded viruses. A significantly improved effect was obtained in *S. littoralis* larvae (Fig. 4). In *H. armigera*, the additive effect was small yet it was significant with vAcLqIT1-IT2 but not with vAcLqαIT-IT2 (Fig. 3). This difference in susceptibility between the two lepidopteran larvae may be associated with variations at the sodium channel target sites, as was shown for scorpion toxins in other insect sodium channels [22].

An analogous approach has been documented for spider and sea anemone toxins, but surprisingly the toxin cooperativity did not yield a major improvement in baculovirus LT_{50} [24]. It is not clear whether the lack of effect could perhaps be due to low expression of one of the toxins. Our constructs expressed both toxins (Fig. 2) and we observed improved insecticidal activity regardless of whether expression was driven by either one or two baculoviruses.

Our results pave the way to further examination of various toxin combinations whose expression is controlled by other temporally regulated viral promoters. Here, we examined the time to paralysis and death of the larvae. Still, other parameters, such as viral yield, that could have significant effect on recombinant virus fitness in the field, require further investigation [25].

The inclusion of an α-toxin, was meant to test the concept of synergism, however, it is clear that elimination of the anti-mammalian toxicity of α-toxins is required prior to any practical utilization [6]. Our results suggest that recombinant baculoviruses may provide an alternative means to hazardous chemicals in insect control, and also demonstrate that further improvement in efficacy is obtainable.

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