

Baculovirus-mediated expression of a scorpion depressant toxin improves the insecticidal efficacy achieved with excitatory toxins

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Abstract The insecticidal efficacy towards *Helicoverpa armigera* lepidopteran larvae of recombinant *Autographa californica* M nucleopolyhedroviruses, expressing depressant and excitatory scorpion anti-insect selective toxins, was investigated. The ET₅₀ (effective paralysis time 50%) values obtained with the recombinant viruses expressing the depressant toxin, LqhIT2, and the excitatory toxin, LqhIT1, were 59 h and 66 h, respectively, whereas the ET₅₀ value of the wild-type virus was longer, 87 h post infection. The insecticidal effects obtained when using two distinct temporally regulated viral promoters revealed advantage for the very late p10 promoter over the p35 early promoter. The higher insecticidity of the virus expressing the depressant toxin compared to the excitatory toxin suggests that pharmacokinetic factors and/or promoter efficiency may play a role during infection of insect pest larvae by recombinant baculoviruses.

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Key words: Depressant anti-insect toxin; Excitatory anti-insect toxin; Baculovirus; Insect cell; Lepidopteran larva

1. Introduction

Baculoviruses are pathogens of invertebrates in general and of insects in particular. Among them nucleopolyhedroviruses (Baculoviridae: subgroup A) infective to Lepidoptera are used as protein expression vectors (reviewed in [1]) and for insect pest control [2,3]. In order to further minimize the damage to crops and reduce the overuse of non-specific chemical insecticides further improvement has been sought. The insecticidal efficacy of baculoviruses (time required to kill the insect) has been enhanced by 30–40%, when their pathogenicity was combined with expression of anti-insect toxins [4–7]. The scorpion excitatory toxin AaIT binds exclusively to sodium channels of insect nervous tissues [8] with high affinity ($K_d = 1–3$ nM) and low capacity (1.2–2.0 pmol/mg protein). AaIT has been considered the most potent anti-insect specific scorpion neurotoxin due to its immediate contraction effect on fly larvae upon injection [9]. Hence, this toxin has become a primary target for engineering baculoviruses [4,6]. Another type of scorpion anti-insect specific toxins, that kill fly larvae at comparable LD₅₀ values to those of excitatory toxins, is the depressant toxin LqhIT2 [10]. Upon injection of blowfly larvae, these toxins induce a short, transient phase of contraction followed by a prolonged flaccid paralysis [11,12]. Its mechanism of action involves depolarization of the axonal membrane,

blockage of the evoked action potential, and changes in the amplitude and the kinetics of the sodium current [13]. Their flaccid properties are attributed to the opening of sodium channels unabling them to inactivate [13]. LqhIT2 was shown to have two non-interacting binding sites on locust neuronal membranes: one of high affinity ($K_d = 1$ nM) and low capacity ($B_{max} = 0.1$ pmol/mg) and another of low affinity ($K_d = 185$ nM) and high capacity ($B_{max} = 10$ pmol/mg). The high affinity site is the target for binding competition of the excitatory anti-insect toxin, AaIT [14,15]. The need for recombinant baculoviruses with improved speed of kill of insect pest larvae motivated us to compare the insecticidal efficacy of baculovirus expressing depressant vs. excitatory neurotoxins. Since the activity of viral promoters is temporally regulated, we also compared the efficacy of two recombinant baculoviruses expressing the excitatory toxin under the control of the early p35 and the very late p10 promoters. Our results indicate that the insecticidal efficacy of *Autographa californica* M nucleopolyhedrovirus (AcMNPV) producing the depressant toxin was significantly improved compared to the efficacy achieved with the expressed excitatory toxin.

2. Materials and methods

2.1. Cell lines and viruses

Spodoptera frugiperda SF9 and BTI-TN-5B1-4 cells were maintained and propagated in TNM-FH medium supplemented with 10% heat inactivated fetal bovine serum [7,16]. Infection of the cells with wild-type AcMNPV strain E2 [17], and plaque titration of virus stocks were as described previously [18].

2.2. Construction of recombinant viruses

The LqhIT2 [19] and LqhIT1 [20] cDNAs, coding for the depressant and excitatory anti-insect-selective toxins, respectively, were engineered via PCR at their 5'- and 3'-termini using oligonucleotide primers. Primer-1, 5'-CCGGATCCAAGATGAAAC(C/T)TATT(G/A)CTTTA-3', for the N-terminal leader sequence, and primer-2, 5'-CCGGATCC(C/T)TAACCGCATGT(A/G)TT(G/T)GTT-3', for the complementary strand at the region encoding the C-terminus, were used to reconstruct LqhIT2-cDNA. Primer-3, 5'-TCTAGAACC-TAGTGGATCC-3', for leader sequence, and primer-4, 5'-CCGGATCCCTAATTAAATTATTGTGAAATC-3', for the complementary strand at the region encoding the C-terminus, were used to engineer LqhIT1-cDNA. The underlined sequences designate *Bam*HI restriction sites that were used for cloning into the transfer vector pAcUW51.P2 (Fig. 1). Thus, two plasmids were obtained, pAcLIT2-pol and pAcLIT1.p10, bearing LqhIT2 and LqhIT1 cDNAs, under the control of the polyhedrin and p10 promoters, respectively. To construct the plasmid pAcLIT1.p35 (Fig. 1), the p35 promoter sequences were PCR-amplified from plasmid pBB/BSst [21] using primer-5, 5'-CCGGATCCTCGGTGCGTACACGTAGTA-3', and primer-6, 5'-TACCGGAAAAATTAGATCTATTTTGCTATG-3'. The reconstructed DNA fragment was cloned back-to-back upstream of the polyhedrin gene in plasmid pI1 [7]. The engineered LqhIT1-

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cDNA was cloned downstream of the p35 promoter in the newly created *Bgl*II site, underlined in primer 6 (Fig. 1). Organization and DNA sequences of the resulting plasmids were confirmed by DNA sequencing [22]. Recombinant viruses were isolated by co-transfecting SF9 cells with the transfer vectors and with linearized polyhedra-negative AcMNPV DNA using the calcium-phosphate method [23]. Polyhedra-positive recombinant viruses, AcLIT2.pol, AcLIT1.p10 and AcLIT1.p35, were isolated by three rounds of plaque purification and verified by restriction enzyme and PCR analyses of the viral DNA.

2.3. Expression, N-terminus sequencing, and biological assays

Production of the toxins in recombinant virus-infected BTI-TN-5B1-4 cells was detected immunochemically [7] using rabbit anti-LqhIT2 and anti-LqhIT1 sera.

Metabolic labeling was performed by adding 40 μ Ci of a [35 S]methionine/cysteine mixture (specific activity 1200 Ci/mmol, New England Nuclear, UK) to methionine/cysteine-deficient Grace medium for an incubation period of 1 h. Then, the medium was replaced, and after 1 h (chase) cell extracts were analyzed by SDS-PAGE and autoradiography [24]. For protein sequencing, the toxin bands were run on a high resolution polyacrylamide gel (18%), transferred onto a PVDF membrane (Millipore Co.), and treated as described previously [7]. Wild type or recombinant baculovirus infections of 1st instar *Helicoverpa armigera* larvae were performed by injection of budded viruses, or by the oral route using polyhedral inclusion bodies as described previously [7].

3. Results

3.1. Expression of LqhIT1 and LqhIT2 by recombinant AcMNPVs

BTI-TN-5B1-4 *Trichoplusia ni* insect cells were infected with the recombinant budded viruses AcLIT2.pol, AcLIT1.p10, and AcLIT1.p35. Expression of LqhIT1, controlled by the very late promoter p10, and that of LqhIT2, controlled by the very late promoter polyhedrin, is shown in Fig. 2.

The de novo synthesis of the depressant toxin LqhIT2 was followed by metabolic labeling using a mixture of [35 S]methionine and cysteine (see Section 2), accompanied by immunodetection of the labeled product (Fig. 3A and 3B, respectively). LqhIT2 synthesis was first detected at 24 h post infection (Fig. 3A, lane 2 and Fig. 3B, lane 2). An increased rate of synthesis of LqhIT2 was observed between 36 to 48 h post infection (Fig. 3A, lanes 3 and 4 respectively). Also, it can be seen that LqhIT2 accumulated in the infected cells at 48 h post infection (Fig. 3B, lane 4). The LqhIT2 product appeared as a double band on high resolution polyacrylamide gels (Fig. 2C). Amino acid sequence analysis determined the slower running band to be the precursor of LqhIT2, i.e. with its intact leader peptide, while the faster running band was the mature toxin (Fig. 3C). It is possible that at late stages of baculovirus infection the secretory pathway of the host is negatively affected [25], or perhaps saturated. This may explain the two forms appearing in the cell extracts.

The activity of the toxins produced by the recombinant baculoviruses was also assessed by injection of 3rd instar *S. littoralis* larvae with the AcLIT2.pol, AcLIT1.p10, and AcLIT1.p35 budded viruses (BVs). Three to four days post injection, characteristic paralysis induced by both toxins was observed regardless of the recombinant virus injected. Western blot analysis of the infected larvae hemolymph revealed a detectable amount of toxin expressed under the control of the very late promoters, whereas LqhIT1 could not be detected when the expression was driven by the early p35 promoter

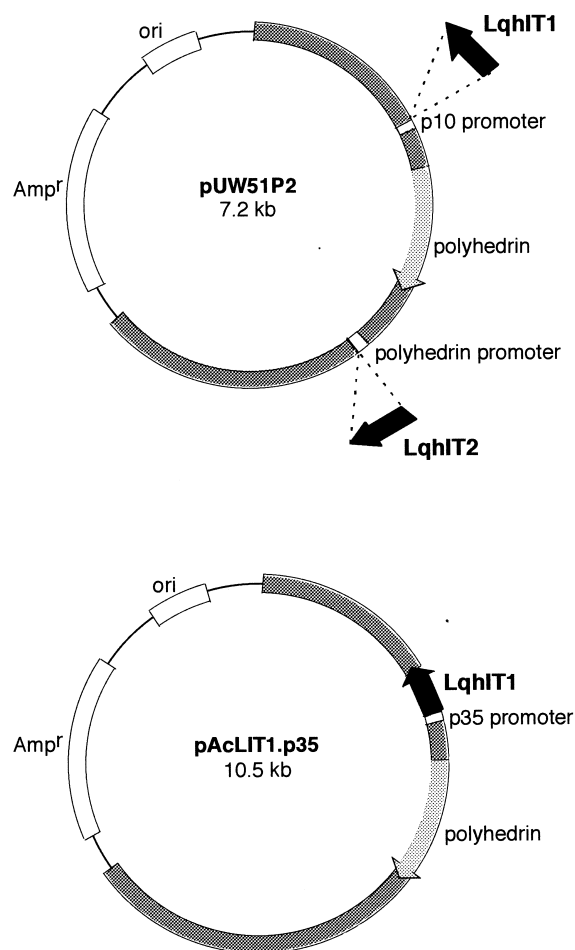


Fig. 1. Construction of transfer vectors utilized to isolate the recombinant baculoviruses. The toxin cDNAs (black arrows) were cloned juxtaposed to the p35, p10 and pol (polyhedrin) promoters yielding the vectors pAcLIT1.p35, pAcLIT1.p10 and pAcLIT2.pol, respectively (see Section 2).

(under our experimental conditions detectable LqhIT1 quantities exceeded 100–150 ng of toxin on Western blots).

3.2. Depressant vs. excitatory paralysis induced by baculovirus infection

The ‘time to paralysis’ of 1st instar *H. armigera* fed with the recombinant baculoviruses (measured by immobility of the larvae) was determined. AcLIT2.pol, expressing the depressant toxin, provided a significantly lower ET_{50} value, 59 h, than that calculated for AcLIT1.p10 expressing the excitatory toxin, 66 h (Fig. 4A and Table 1). The ET_{50} values of AcLIT1.p35 infections were 73 h (Fig. 4 and Table 1). Thus, the p10-driven LqhIT1 expression induced faster paralysis than the p35-driven LqhIT1 expression (Fig. 4B and Table 1).

ED_{50} values obtained were of 0.9×10^3 , 1.1×10^3 , 2.2×10^3 , for the recombinant AcLIT2.pol, AcLIT1.p10, and AcLIT1.p35, respectively, vs. 1.4×10^3 for the wild type AcMNPV (Table 1).

Evidently, the baculovirus expressing the depressant toxin showed improved insecticidal efficacy (faster paralysis) compared to the virus expressing the excitatory toxin.

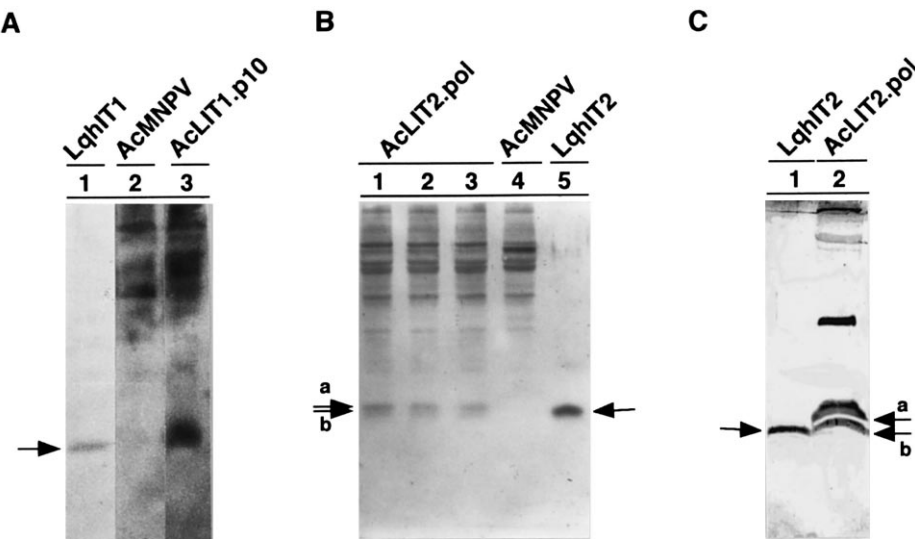


Fig. 2. AcMNPV-mediated expression of LqhIT2 and LqhIT1 anti-insect toxins in insect cells. BTI-TN-5B1-4 cells were infected with AcLIT1.p10 (A, lane 3) and AcLIT2.pol (B, lanes 1–3, indicating expression from three different isolates and C, lane 2) recombinant viruses or with wild type AcMNPV (A and B, lanes 2 and 4, respectively). Cell extracts at 48 h post infection were subjected to SDS-PAGE and immunoblot analysis using anti-LqhIT1 (A) and anti-LqhIT2 (B and C) antisera. LqhIT1 and LqhIT2 markers are indicated (A, lane 1, and B and C, lanes 5 and 1, respectively). C: High resolution SDS-polyacrylamide gel. Arrows a and b, band of LqhIT2 toxin.

4. Discussion

Biotechnology has already proven useful in generating novel biopesticides aiming at reducing current overuse of hazardous insecticidal chemicals [26]. The restricted host range of baculoviruses and their ability to penetrate upon feeding into the internal tissues of lepidopterous pests, make them ideal vectors for mobilizing selective toxins into target insects

with minor predicted effects on the environment. This approach has been recently demonstrated with AcMNPVs bearing various arachnid toxin genes [4–7]. Among scorpion toxins, the excitatory toxin, AaIT, has been the focus of these experiments, since it is considered as the most potent anti-insect specific scorpion neurotoxin [9]. Despite its high toxicity upon injection to fly larvae, locusts, some beetles and isopods [27], this toxin, as well as other scorpion toxins, shows weak

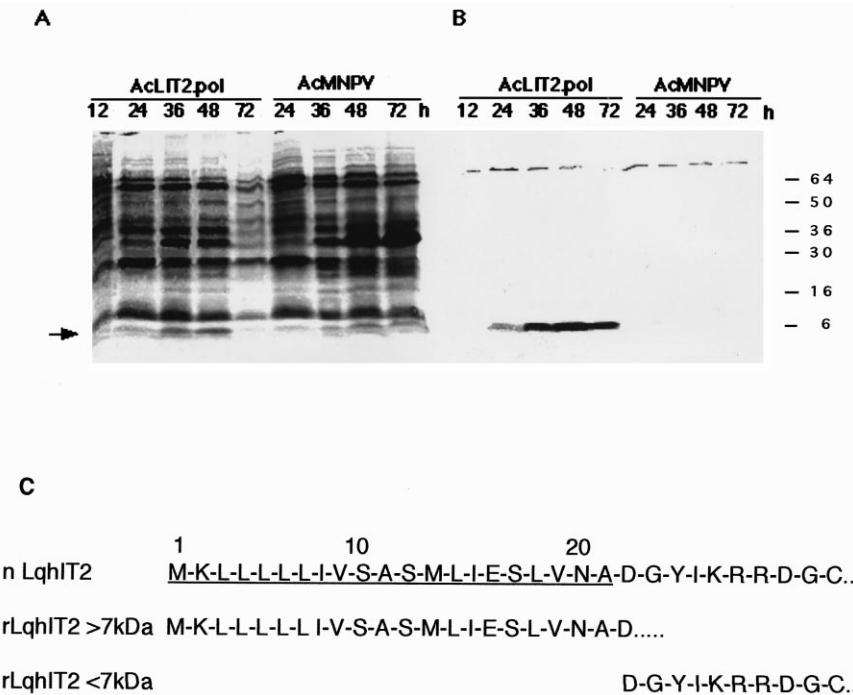


Fig. 3. Time-course expression of LqhIT2 in insect cells. A: ³⁵S-amino acid metabolic labeling of AcLIT2.pol and AcMNPV-infected cells. B: Immunoblot analysis. C: N-terminal amino acid sequence of the natural (n) and recombinant (r) LqhIT2 toxins. Time (h) post infection is indicated above each lane. M, size marker, values (kDa) indicated on the right hand side. rLqhIT2 > 7 kDa and rLqhIT2 < 7 kDa in C, correspond to the upper and lower bands seen in Fig. 2B and C (arrows a and b), resolved and identified as described in Section 2. Underlining in nLqhIT2 corresponds to the leader peptide.

and delayed effects when injected to *Spodoptera* or *Helicoverpa* larvae. This phenomenon suggested either possible barriers unabling the toxins to reach their receptor sites, or enhanced metabolic degradation in these noctuid larvae. Strikingly, these toxins are very effective in the context of baculovirus mediated expression [4–7]. The tracheal system of the host was shown as a major conduit of baculovirus infections [28]. Thus, it is conceivable that expression of the scorpion toxins adjacent to their target sites in motor neural tissues overcomes pharmacokinetic barriers and may be responsible for their enhanced effects when compared to injection experiments of purified toxins. Support for this suggestion may be provided by the results obtained with LqhIT1 expression. We were able to detect a clear paralytic effect using the p35 promoter, comparable to the p10-mediated expression of this toxin, although we could not detect the toxin immunochemically in the hemolymph. It seems that a small amount of toxin is required for such an effect when its expression is directed by a baculovirus vector. It was also reported by McCutchen et al. [6] that very low amounts, 1–2 ng/ μ l, of baculovirus-expressed AaIT were found in the hemolymph of paralyzed *Heliothis virescens* larvae. These results show great improvement over those obtained by injection of pure AaIT [29] and recombinant LqhIT2 (unpublished).

Although the excitatory toxin generates an immediate paralytic effect in fly larvae in contrast to the delayed effect of the depressant toxin, the baculovirus-driven expression of these toxins in lepidopterous larvae, reveals an opposite phenomenon (Fig. 4 and Table 1). This effect could occur due to:

1. Pharmacokinetic differences between excitatory and depressant toxins. If depressant toxins expressed by AcMNPV in noctuid larvae are more stable than excitatory toxins, their bioactivity may be better pronounced and explain the improved insecticidal efficacy of AcLIT2.pol vs. AcLIT1.p10.
2. Differences in promoter efficiency. p10 and polyhedrin promoters are both very late strong promoters, but may not be equally active.

Table 1

Effective time- and dose-paralysis/death response of first instar *H. armigera* larvae infected with recombinant and wild type baculoviruses

Virus	Time to paralysis and/or death response (95% fiducial limits) ^a			
	ET ₅₀ ^a	Lower	Upper	Slope
AcLIT2.pol	59	51	66	6.06
AcLIT1.p10	66	61	71	8.02
AcLIT1.p35	73	69	77	7.11
AcMNPV	87	76	98	9.10
	Dose to paralysis and/or death response (95% fiducial limits) ^b			
	LC ₅₀ ^b	Lower	Upper	Slope
AcLIT2.pol	0.9×10^3	0.7×10^3	1.1×10^3	2.63
AcLIT1.p10	1.1×10^3	0.8×10^3	1.5×10^3	2.09
AcLIT1.p35	2.2×10^3	1.5×10^3	3.4×10^3	1.42
AcMNPV	1.4×10^3	0.9×10^3	2.0×10^3	1.40

^aTime in hours when larvae were placed on the infected diet.

^bPolyhedra per mm² of contaminated diet. LC₅₀ and ET₅₀ values were calculated using probit analysis [30].

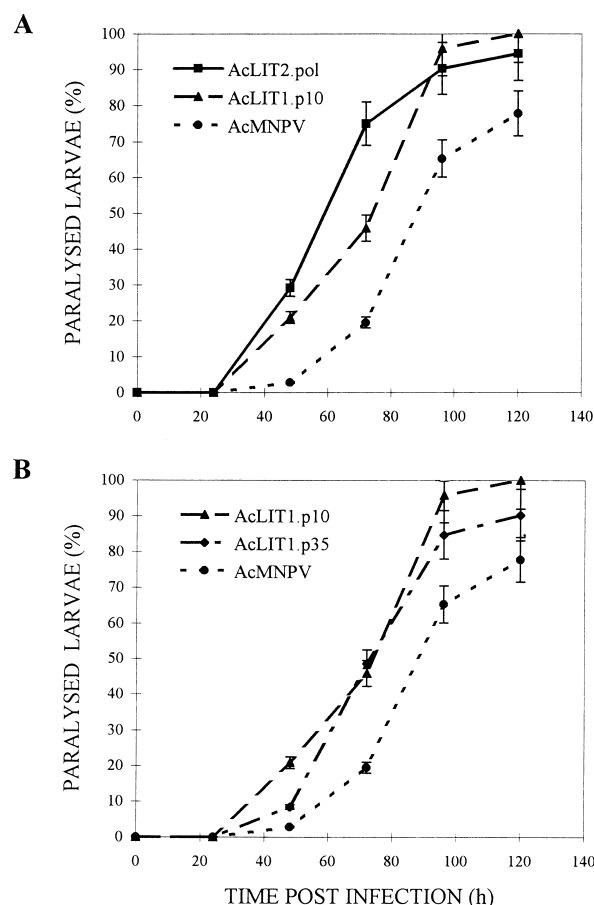


Fig. 4. Time (hours) to paralysis and/or death-response of orally infected *H. armigera* first-instar larvae. A: Bioassay with AcMNPV expressing depressant (LqhIT2) vs. excitatory (LqhIT1) anti-insect toxins (AcLIT2.pol and AcLIT1.p10, respectively). AcMNPV, wild type virus. B: Bioassay of AcLIT1.p35 and AcLIT1.p10 expressing LqhIT1 under the control of early and very late baculovirus promoters p35 and p10, respectively. AcMNPV, wild type virus. Dose per treatment was 4×10^3 PIBs/mm².

Tomalski and Miller suggested that the concentration of the toxin and its early appearance during infection, should be considered to further improve the efficacy of baculoviruses [5]. The results obtained when utilizing the p10 and p35 promoters (Table 1 and Fig. 4) support this hypothesis.

Finally, our results suggest, that the insecticidal efficacy of a given scorpion toxin should be evaluated *in vivo*, e.g. by expression of baculoviruses, and cannot solely rely on its paralytic effects observed upon injection of test animals such as blowfly larvae.

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