Functional expression of an alpha anti-insect scorpion neurotoxin in insect cells and lepidopterous larvae

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Abstract **The *Leiurus quinquestriatus hebraeus* alpha anti-insect toxin (LqhαIT) cDNA was engineered into the *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) genome. Insect cells infected with the recombinant virus secreted a functional LqhαIT polypeptide. *Spodoptera littoralis* and *Heliothis armigera* larvae injected with recombinant budded virus, showed typical intoxication symptoms. This recombinant virus showed enhanced insecticidal potency against *H. armigera* larvae compared with wild type AcNPV. The present expression system will facilitate: (1) the future elucidation of structural elements involved in its prominent anti-insect toxicity; and (2) the future design of genetically modified alpha toxins with improved anti-insect selectivity.**

Key words: LqhαIT (*Leiurus quinquestriatus hebraeus*) anti-insect toxin; Baculovirus; Insect cells; Lepidopterous larvae

1. Introduction

The various polypeptide neurotoxins from *Buthinae* scorpion venoms which affect sodium conductance, can be subdivided mainly into: (a) anti-insect selective toxins such as the excitatory and depressant toxins which exclusively affect insects [1], and (b) non-selective neurotoxins such as the alpha toxins [1]. The alpha toxins are responsible for human envenomation by scorpion venoms [2] but they affect insects as well [1]. An alpha toxin, LqhαIT, recently isolated from the venom of the Israeli yellow scorpion *Leiurus quinquestriatus hebraeus* [3] was shown to affect insects strongly and to possess weak toxicity towards mammals. LqhαIT, resembles alpha mammal toxins in its primary structure and ability to slow or even block the sodium current inactivation process in an insect axonal preparation [3]. However, LqhαIT has been shown to bind with high affinity to an insect but not to a mammal neuronal preparation [4].

Thus, LqhαIT is able to distinguish between insect and mammalian sodium channels [3,4] and hence it may serve as an excellent model for the clarification of structural elements that confer high anti-insect specificity. This demands the expression of the corresponding cDNA to enable a genetic approach for its structure–activity relationships. We engineered the *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcNPV) baculovirus with the LqhαIT cDNA [5] under the control of the potent polyhedrin viral promoter. In the present report we describe the successful expression of functional LqhαIT whose secretory leader signal was properly processed by the insect cells. We also demonstrate a significant increase in the insecticidal potency of the recombinant virus, which is of applicative value.

2. Materials and methods

2.1. Engineering of the transfer vector pAcLα22

The LqhαIT-cDNA [5] was engineered via PCR using the primers: I) 5′-CTGTCTGCAGCATTTGGTAATGATTAGTTTGGC-3′, and II) 5′-GAGGATCCTTAGCGGCATTTTCCTGGTACTCT-3′ specific for the upstream region 5′ to the initiator ATG and downstream region 3′ to the terminator TAA of the toxin, respectively. The DNA product was thus provided with a PstI site and a BamHI site at the gene 5′ and 3′ ends respectively (underlined sequences). The additional codons at the carboxy terminal side, which are normally removed by processing, were omitted.

The *Eco*RI I fragment of AcNPV, containing the entire polyhedrin gene sequence as well as the 5′ upstream and 3′ downstream viral sequences [6], was cloned into the phagemid pTZ18U (USB), thus resulting in plasmid pI1. The initiator ATG of the polyhedrin gene was constructed within an *Nsi*I site by site-directed mutagenesis [7]. Digestion of pI1 with *Nsi*I and *Bam*HI provided compatible ends to the *Pst*I and *Bam*HI digested LqhαIT cDNA clone, thus yielding a polyhedrin-negative plasmid pAcLα1. A polyhedrin-positive version of it, pAcLα22 was constructed by digesting pAcLα1 with *Bst*UI and subcloning the fragment bearing the LqhαIT cDNA under the control of the polyhedrin promoter, into the *Eco*RV site located upstream to the 5′ end of the existing polyhedrin gene of the original pI1 (Fig. 1). Orientation and sequence of the engineered toxin cDNA were confirmed by restriction enzyme analysis and sequencing [7]. Plasmid propagation and transformations in *E. coli* strain MV1190 were performed using standard procedures [7].

2.2. Isolation of the recombinant virus AcLα22

pAcLα22 and polyhedrin negative AcNPV DNA [8] were co-transfected into *Spodoptera frugiperda* cells (Sf9) (grown in TNM-FH medium supplemented with 10% fetal bovine serum [6]). Recombinant viruses were isolated after three cycles of plaque purification in Sf9 cells [6,8].

2.3. Expression of LqhαIT

2.3.1. Expression of LqhαIT in: (1) *Trichoplusia ni* BTI-TN-5B1-4 cells [9] were grown in TNM-FH medium as indicated above, and infected with recombinant virus at a multiplicity of infection of 10 (3×10^5 cells). Cell supernatants were collected at various intervals post-infection (p.i.) and clarified from cellular debris by centrifugation; aliquots were assayed for toxicity on *Sarcophaga falculata* blowfly larvae or, used as a source of budded virus (see below). The cell pellets were washed in phosphate saline buffer, resuspended in Tris 250 mM, pH 7.5, EDTA 1mM, Aprotinin 1%, subjected to three cycles of freezing and thawing, and centrifuged at $10000 \times g$ for 15 minutes at 4°C. LqhαIT was monitored in the cell extracts (see below).

2.3.2. Expression of LqhαIT in: (2) *H. armigera* and *S. littoralis* larvae infected with the recombinant virus. Hemolymph was collected

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on ice (from an incision in the larva proleg) and immediately mixed with 0.5 volume of SDS-PAGE sample buffer.

2.4. Immunodetection of LqhαIT

Samples of cellular extracts or insect hemolymph were analyzed by gel electrophoresis [10]. The gel was blotted onto a nitrocellulose membrane (Hybond-C, Amersham). Detection of the toxin was performed with anti-LqhαIT serum, followed by incubation with an anti-rabbit IgG antibody conjugated to alkaline phosphatase, using 5-bromo-4-chloro-3′-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) as substrates [7].

2.5. Bioassays

Samples (supernatants or cellular extracts) were injected into 100-mg body-weight *S. falculata* larvae. Sustained contraction paralysis at 5 min post-injection indicated the presence of active alpha insect toxin [3].

The biological activity of the recombinant viruses was assayed on lepidopterous 3rd instar larvae (100 mg body weight) injected with 5×10^5 plaque forming units (p.f.u.) budded virus (1–5 μl), placed on synthetic diet and monitored daily for the appearance of intoxication symptoms. Polyhedral inclusion bodies (PIBs) were isolated by lysing the infected cells in Tris 10 mM pH 7.3, SDS 1%, EDTA 1 mM, followed by washing and resuspending in phosphate saline buffer; they were then counted in a hemocytometer and diluted to the desired working concentration. Oral infection of 2nd instar larvae (24 animals per sample) was performed by contamination of diet plugs with PIBs [11]. Dose-mortality curves were derived as described previously [12]. The toxicity assays were performed at 27°C and monitored daily.

2.6. Purification and N-terminal sequence of the recombinant LqhαIT

Supernatant of AcLα22-infected cells collected at 72 h. post-infection was dialyzed (cut-off 3500 Da) and lyophilized. The dried material was subjected to three successive chromatography steps: (a) gel filtration on Sephadex G-50 fine (Pharmacia); (b) cation-exchange chromatography on CM-52 cellulose (Whatman); and (c) reverse-phase HPLC on RP-C_{18} column (4.6 × 250 mm, Vydac). The amino acid sequence was performed by automated Edman degradation using an Applied Biosystem gas-phase sequencer connected to its corresponding PTH-analyzer and data system according to a previously described [3] method.

3. Results

3.1. Engineering of a recombinant baculovirus bearing the LqhαIT-cDNA

The LqhαIT-cDNA was cloned into an AcNPV-based, polyhedrin-positive, transfer vector (Fig. 1). The resulting vector, pAcLα22, retained intact the 5′ end of the polyhedrin gene where the polyhedrin ATG initiator triplet was fused with the first ATG codon in the leader peptide of the toxin. Thus, the LqhαIT gene remained under the control of the polyhedrin promoter, in the opposite direction to the existing polyhedrin gene (Fig. 1). The plasmid, pAcLα22 was used to generate the recombinant virus AcLα22 (see section 2).

3.2. Expression of the LqhαIT in insect cells

BTI cell monolayers were infected with the recombinant virus AcLα22, and the 7.5-kDa LqhαIT toxin was first detected by immunoblot after 48 h p.i.; its level had increased at 72 and 96 h. p.i. (Fig. 2A). No toxin was produced in wild type AcNPV-infected cells (Fig. 2A).

Table 1
Biological activity of the recombinant LqhαIT derived from AcLα22-infected cells at various times post-infection

	LqhαIT (PU/3.3 × 10^5 cells)[a]			
Days post-infection	1	2	3	4
Supernatant	0	84	140	140
Cell-associated	0	60	120	120

[a] Aliquots of extracts or supernatants of AcLα22-infected BTI-TN-5B1–4 cells were injected into *S. falculata* larvae (100 mg body weight).

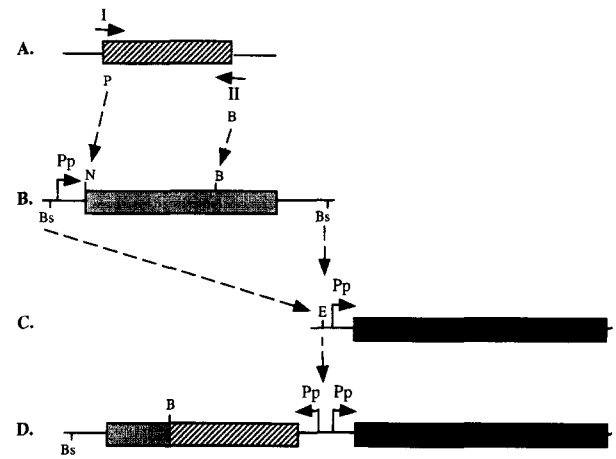

Fig. 1. Cloning of LqhαIT cDNA into a baculovirus transfer vector. The toxin cDNA (hatched bar) was amplified by PCR using the primers I and II (A), restricted with *Pst*I (P) and *Bam*HI (B) enzymes and ligated to the baculovirus transfer vector (B) restricted with *Nsi*I (N) and *Bam*HI (thus remaining under the control of the polyhedrin promoter (Pp) by replacing the polyhedrin coding sequences). The resulting plasmid was further restricted with *Bst*UI (Bs) and cloned into the *Eco*RV (E) site of the polyhedrin gene positive (black bar) transfer vector (C) to give the polyhedrin-positive (transfer vector (D).

3.3. Biological activity of the recombinant toxin and virus

Activity of the recombinant LqhαIT was monitored by injecting extracts of AcLα22-infected cells into blowfly larvae and comparing their effects with that of the purified toxin. The characteristic paralysis of the blowfly larvae [3] was used to determine the amount of active toxin secreted during the infection, which was estimated as 140 paralytic units/3.3 × 10^5 cells at 48 h. p.i. (Table 1). Infection of 3rd instar *Spodoptera littoralis* larvae by injection of the recombinant budded virus (AcLα22) induced after three days progressive contractions of the body musculature and uncoordinated leg movements, accompanied by the appearence of tiny droplets of liquid on the surface of their integument, and followed by shrinkage and body weight loss. The subsequent paralysis ended in lethality. Fig. 2B, shows the inability of the virus-infected larvae to respond to physical touch with a microtip. None of these symptoms were observed with larvae injected with wild type AcNPV. The presence of LqhαIT in the larvae hemolymph was confirmed immunochemically by anti-LqhαIT serum (Fig. 2C). Similar results were observed with *Heliothis armigera*-injected larvae (data not shown).

3.4. Insecticidal effects of the recombinant viruses

The insecticidal potency of recombinant and wild type viruses was compared by means of a quantitative bioassay in which *Heliothis armigera* larvae were orally infected with 4000 polyhedrin inclusion bodies (PIBs) of AcLα22 and AcNPV, respectively. Lethal time 50% (LT_{50}) was calculated from time–mortality curves (Fig. 3, top) for both viruses; they corresponded, to approximately 78 and 120 h p.i., for AcLα22 and AcNPV, respectively. Similarly lethal dose 50% (LD_{50}) values for the recombinant and wild type viruses, determined at 164

h p.i., corresponded to 40 and 45 (± 10%) PIBs respectively. The ability of the virus-infected larvae to feed was determined by measuring their weight gain as a function of time. As shown in Fig. 3 (bottom), gain in weight of *Heliothis* larvae ceased after 90 h of ingestion of AcLα22 polyhedral bodies. AcNPV-infected larvae still gained weight after 120 h p.i. (the mean weight of the latter population, 130 ± 5 mg, was 3 times higher than that of the recombinant virus-infected larval population).

3.5. *Purification and determination of the N-terminal sequence of the recombinant LqhαIT*

As shown in Table 2 and Fig. 4 the recombinant toxin was purified from the supernatant of AcLα22-infected cells by three successive column chromatography steps (gel filtration, ion exchange and reverse-phase high performance liquid chromatography).

As shown (Table 2) the final product revealed a 26,000-fold increase of purification and specific toxicity of the authentic chemically purified toxin [3]. The 0.01 $O.D._{50}$ unit obtained in the final purification step corresponds, according to the extinction coefficient ($E^{1\%}_{1cm}$ 280 nm) to 5μg, shown to possess 14 ng as a toxicity unit to blowfly larvae [3]. In spite of the high increase in specific activity, the recovered toxicity did not exceed 10% (Table 2). However, we have no doubt that this toxicity represents the pure and identified recombinant LqhαIT toxin as assessed by immunoblotting, electrophoresis, amino acid analysis (data not shown) and amino acid sequence determination (Fig. 4), which also confirmed that the leader sequence of the pre-toxin was correctly processed by the insect cells, generating the precise N-terminus as found in the authentic toxin.

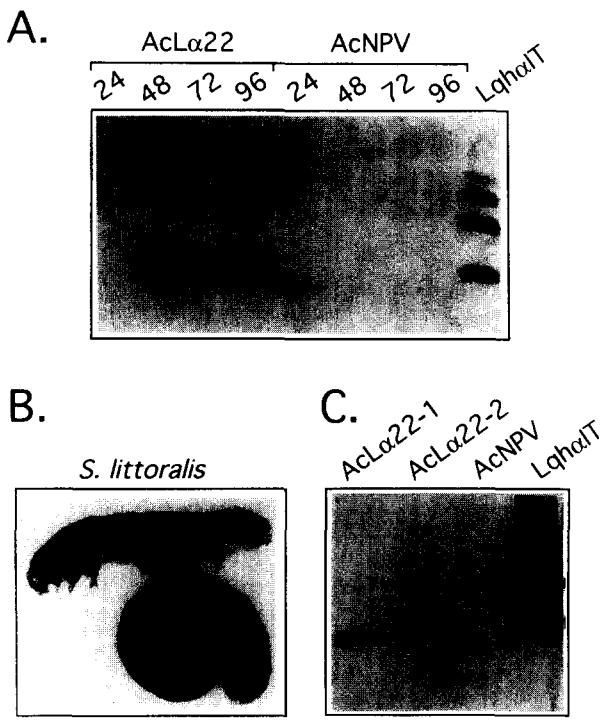

Fig. 2. Expression of LqhαIT. (A) BTI-TN-5B1–4 cells were infected with recombinant (AcLα22) or wild type (AcNPV) viruses, respectively. Extracted aliquots at 24, 48, 72 and 96 h p. i. were subjected to SDS-PAGE. The gel was blotted onto nitrocellulose and probed with anti-LqhαIT serum. LqhαIT lane, control toxin. (B) Response of *S. littoralis* larvae infected by AcLα22 (upper) and AcNPV (lower) to a microtip touch. (C) Western blot analysis, as in A, of hemolymph extracted from *S. littoralis* larvae infected with AcLα22 (two separate animals, AcLα22-1 and AcLα22-2 lanes) and wild type (AcNPV lane). The bands larger than the smaller 7.5-kDa band present in control and recombinant LqhαIT are due to aggregation of the polypeptide monomer and correspond to molecular weights of 15, 30 and 60 kDa.

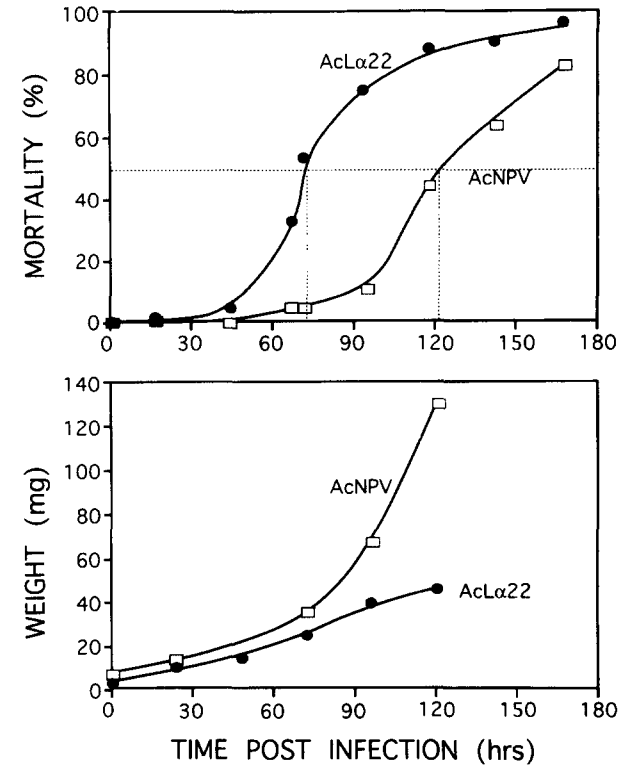

Fig. 3. Oral infectivity bioassay by recombinant (AcLα22) or wild-type (AcNPV) viruses to *H. armigera* larvae. (A) Mortality curve. (B) Mean weight curve. A ± 10% deviation of the recorded values in A and in B was observed.

4. Discussion

Our data show that the alpha scorpion toxin, LqhαIT, was expressed in a baculovirus-based eukaryotic system. The identi-

Table 2
Purification of the recombinant LqhαIT toxin

Separation method	Protein contents (OD_{280} units)	Toxicity to blowfly larvae (ED_{50} units)	Degree of purification (fold)
Starting material, supernatant of infected cells (72 h p.i.)	270	3,750	1
Sephadex G-50 (see Fig. 3A)	2.8	2,860	77
CM-cellulose cation exchange column (2.5 × 0.5 cm 0.2 M amm. acet. pH 6.7 buffer)	0.1	1,600	1,206
C_{18}-RP-HPLC (see Fig. 4B)	~0.01 (5 μg)	350	26,000

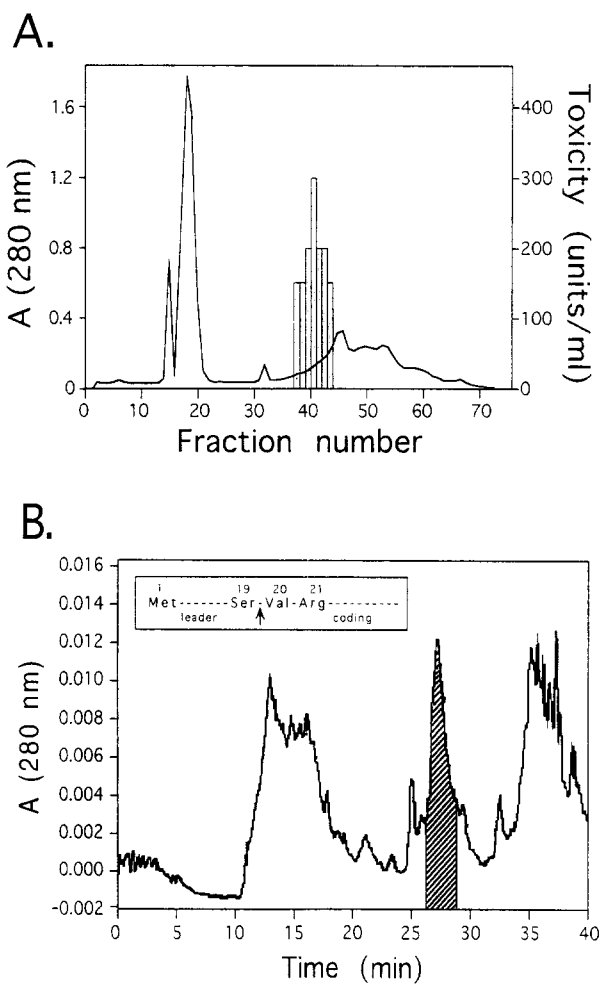

Fig. 4. Purification of recombinant LqhαIT. (A) Gel filtration through Sephadex G-50 (110 × 1.5 cm) in 10 mM ammonium acetate buffer, pH 6.7. Flow rate was 24 ml/h; 3 ml fractions were collected. Samples of 5μl of each fraction were injected into blowfly larvae. The fractions containing toxicity are marked by dashed lines. (B) HPLC separation of the CM-52 derived fraction on a C_{18} RP column (4.6 × 250 mm, Vydac). Buffers: A, 0.1% trifluoroacetic acid (TFA); B, 0.1 TFA in acetonitrile. Gradient: 0 min, 100% buffer A; 10 min, 23% buffer B; 25 min, 28% buffer B; 35 min 100% buffer B; 40 min, 100% buffer B. Flow rate was 1 ml/min. The toxic fraction is indicated by cross-hatched lines. Inset: amino acid residues of the recombinant toxin leader peptide and coding regions, the arrow indicates the cleavage site and indicated are two (Val and Arg) of the N-terminal 10 residues determined by sequence analysis (residues 19 to 29).

fication of a functional toxin was assessed immunochemically, chemically and by biological assays with blowfly larvae and virus-infected lepidopterous larvae (Figs. 2,3,4 and Tables 1 and 2). Previous studies reported that baculovirus-directed functional expression of the excitatory anti-insect selective scorpion toxin AaIT fused to a signal peptide coding sequences of an insect protein bombyxin or of the baculovirus protein gp67 [12–14]. In the present study, we demonstrate that expression, secretion and correct processing of LqhαIT can be achieved by direct cloning of its cDNA, including the LqhαIT signal peptide coding sequences.

These data should be evaluated from the applicative and pharmacological aspects. From the applicative point of view, it is noteworthy that the AcLα22 recombinant virus significantly enhanced the insecticidal efficacy towards lepidopterous larvae and it possesses the additional technological advantages that it occupies a different binding site on the insect sodium channel from that of the excitatory toxin [2]. This suggests that the alpha insect toxin is a good candidate for engineering double-expression vectors together with the excitatory anti-insect toxin, which may yield a synergistic interaction between the toxins.

However, it should be borne in mind that the LqhαIT toxin lacks absolute selectivity for insects, a fact reflected in intoxication symptoms observed from injection of LqhαIT into laboratory mice [3]. Thus, further studies and genetic modifications should be introduced to LqhαIT prior to considering its utilization for insect pest control (see below).

The pharmacological value of the present expression system lies in the fact that LqhαIT is able to distinguish between insect and mammalian neuronal membranes [2]. Genetic alterations of selected amino acid residues may result in an alpha toxin which affects insects but not mammals.

The present baculovirus system provides the necessary technological means to achieve this goal.

The above approach will enable to reach two important objectives: i) the development of a pharmacological tool for the study of the structure-function relationships of insect sodium channels; and ii) the design of anti-insect toxins for the improvement of insect-pest control.

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