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Utilization of scorpion insecticidal neurotoxins and baculoviruses for the design of novel selective biopesticides

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1. Natural toxins suitable for insect pest control

Public concern over the risks associated with widespread use of insecticidal chemicals has motivated our efforts to develop safer and more effective approaches to pest control. Thus, natural insecticidal compounds derived from venomous animals may serve as rational alternatives. Venoms of arthropods such as scorpions, spiders, braconid and sphecid wasps, reduviid bugs and centipedes (Zlotkin, 1985) which prey on insects, possess anti-insect selective polypeptidic neurotoxins. Such toxins have already shown promise when incorporated into insect pathogens by enhancing their killing efficacy (Chejanovsky *et al.*, 1995; Maeda *et al.*, 1991; Stewart *et al.*, 1991; Tomalski and Miller, 1991). These toxins bind to insect sodium channels (Gordon *et al.*, 1992), modify their properties and disrupt normal neuromuscular functions leading to paralysis and death. Thus, the incorporation of anti-insect selective scorpion toxins into insecticidal microorganisms offers an opportunity to improve and augment current crop protection strategies (Maeda *et al.*, 1991; Stewart *et al.*, 1991; Tomalski and Miller, 1991). In order to minimize biological hazards which may be associated with such a genetic approach and to avoid the possibility of resistance build-up of insect pests, the molecular basis for anti-insect selectivity of these toxins should be clarified. Furthermore, usage of insecticidal toxins should be limited to those revealing the 'animal group specificity' phenomenon where a toxin shows activity against a given group of organisms and is not effective against other groups of organisms. In cases where a venomous organism feeds on a given limited group of animals, the 'animal group specificity' is manifested already on the level of the whole venom. For example, venomous marine Conidae snails are subdivided according to their feeding habits into fish, mollusc and worm eaters. A series of early bioassays (Endean and Rudkin, 1963) demonstrated that this feeding preference was associated with the specific toxicity of their venoms aimed at the respective groups of prey animals. An extreme example of anti-insect selectivity revealed by an entire venom (gland extract) is demonstrated by Braconidae wasps. Their venom is employed primarily for securing hosts (moth larvae) for their offspring. The venom is exclusively effective against moth larvae, in

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which it induces a flaccid paralysis of long duration (weeks) as a consequence of a presynaptic blockage of excitatory transmitter (glutamate) release at the neuromuscular junction (Piek and Spanjer, 1986).

A distinction between arthropod affecting and mammal non-affecting scorpion and spider venoms has been made (Zlotkin, 1985). Clear detection of anti-insect specific toxins has been essentially obtained by chemical fractionation of arthropod venoms. Thus, vertical column electrophoresis of the black widow spider (*Latrodectus*) venom distinguished between several protein fractions (Mr = 60–80 kDa), each causing either fast reversible, or slow progressive irreversible paralysis of flies or toxicity to guinea pigs (Frontali and Grasso, 1964). Similarly, molecular exclusion column chromatography coupled to starch gel electrophoresis enabled the isolation of polypeptide factors from scorpion (Buthidae) venoms which selectively affect insects, vertebrates or crustaceans (Zlotkin, 1985, 1988; Zlotkin *et al.*, 1991).

Among the scorpion toxins that show selectivity for insect sodium channels over their vertebrate counterparts, are the excitatory (Zlotkin, 1983) and the depressant (Zlotkin *et al.*, 1993) toxins. The first excitatory anti-insect toxin, AaIT, was isolated in 1971 (Zlotkin *et al.*, 1971) and its specificity was confirmed by bioassays on intact animals, *in vitro* neuromuscular preparations and binding assays to various neuronal membranes. The excitatory toxins are single chained polypeptides of 70 amino acids cross-linked by four disulphide bridges, three of which appear at homologous positions as compared to other toxins affecting sodium channels. They interact with insect neuronal preparations through a single class of high affinity (K_D = 1–2 nM) and low capacity (1.2–2.0 pmol mg^{-1} membranal protein) binding sites. The rapid excitatory paralysis of insects promoted by these toxins is due to the induction of repetitive electrical activity of motor neurons. This excitatory effect is attributed to the increase of the activatable action potential sodium conductance and a voltage-dependent slowing of its turning off (Pelhate and Zlotkin, 1981; Zlotkin, 1985, 1988). The depressant toxins constitute 61–64 amino acid long polypeptides with unique primary sequences; however, their four disulphide bridges are positioned homologously to other scorpion toxins. These toxins reveal a dual mode of action, where a short, transient period of excitability (similar to that obtained with the excitatory toxins) precedes the progressive flaccid paralysis induced by them in insects. The depressant toxins perturb binding of the excitatory toxins to their binding site at the insect neuronal membrane (Zlotkin *et al.*, 1991) although their receptor site was shown to be distinct from that of the excitatory toxins (Moskowitz *et al.*, 1994). The end result of their effect, flaccid paralysis, is a consequence of blockage of the evoked action potentials (Zlotkin, 1985) which may result from two mechanisms: first, suppression of the activatable sodium conductance as revealed by voltage clamp data; and second, depolarization of the axonal membrane attributed to the increase of the resting sodium permeability (Zlotkin, 1985, 1988).

Another group of scorpion toxins, the α-toxins, shows activity against both insects and mammals. This class constitutes a family of structurally and functionally related polypeptides, each containing 62–65 amino acid residues cross-linked by four disulphide bridges (Rochat *et al.*, 1979; Figure 1). Alpha mammal toxins from the

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                10        20        30        40        50        60
                :         :         :         :         :         :
LqhαIT  .VRDAYIAKNYNCVYECFRDAYCNELCTKN.GASSGYCQWAGKYGNACWCYALPDNVPIRVP..GKCR
Lqq4    G-------DDK----T-GSNS---TE---D.--E------L---------IK---K----I-..----
Bot1    G.------QPE------AQNS---D----D.--T------L---------KD--------I-..---HF
Bot11   .LK-G--VDDR--T-F-GTN-----E-V-LK-.E------V-R--------K---H-RTVQA..-R--S
BeM10   .---G---DDKD-A-F-G-N---D-E-K-..--E--K-WY--Q--------K---W---KQKVS---N
Lqq5    .LK-G--VDDK--TFF-G-N----DE-K-K.-GE------ASP--------K---R-S-KEK..-R-N
Amm5    .LK-G--I--L--TFF-------DD--K-K.-G-------ASP----------R-S-KEK..-R-N
AaH2    .---G--VDDV--T-F-G-N-----E---LK-.E------ASP-----Y--K---H-RTKG-..-R-H
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Figure 1. Sequence comparison between scorpion alpha neurotoxins. Identical residues with the LqhαIT sequence are designated by dashes. Lqq4 and Lqq5, *Leiurus quinquestriatus quinquestriatus* toxin 4 and toxin 5; Bot1 and Bot11, *Buthus occitanus tunecatus* toxin 1 and toxin 11; BeM10, *Buthus eupeus* toxin 10; LqhαIT, *Leiurus quinquestriatus hebraeus* alpha insect toxin; Amm5, *Androctonus mauretanicus mauretanicus* toxin 5; AaH2, *Androctonus australis* Hector toxin 2.

scorpion *Androctonus australis* Hector (such as AaH1 and AaH2) or from *Leiurus quinquestriatus quinquestriatus* (such as Lqq4 and Lqq5) bind in a voltage dependent manner to the receptor site of vertebrate voltage sensitive sodium channels (Catterral, 1977; Couraud *et al.*, 1978; Ray and Catterral, 1978). Their toxic effect is due to a modification of the channel activity by slowing inactivation and altering its voltage dependence (Catterral, 1980, 1986; Gordon, 1990). As specific modifiers of the inactivation process, they are practically used as tools for the clarification of the molecular mechanism of ion channel gating. The large repertoire of various α-scorpion toxins is of particular significance as it is useful for the identification, characterization and discrimination between sodium channels in different organisms, tissues, and even in different locations within the same tissue (Barchi, 1987).

Recently, a new α-toxin, LqhαIT, with salient similarity in sequence and in mode of action to the anti-mammal alpha scorpion toxins, was characterized in the venom of the scorpion *Leiurus quinquestriatus hebraeus* (Eitan *et al.*, 1990). This toxin is active against both insect and mammalian sodium channels and its binding site does not overlap with those of either the excitatory or depressant anti-insect selective toxins in reciprocal radioligand binding experiments (Eitan *et al.*, 1990; Gordon and Zlotkin, 1993). LqhαIT thus recognizes a binding site on the sodium channel that may be common to insects and mammals, while at the same time having an apparent higher affinity for the insect channel.

On this basis, our goal to clarify the molecular basis for the anti-insect selectivity of scorpion neurotoxins is focused, in its initial stages, on LqhαIT as a representative of the α-toxin class. There are several advantages in pursuing this study with α-toxins:

- Alpha scorpion toxins comprise a large group of the most studied scorpion neurotoxins displaying natural variability with respect to primary sequences, binding sites and displaceability characteristics, potency, and the ratio of their anti-insect/mammal toxicity.

- Biological assays, including radioligand binding and neurophysiological assays for insect and mammalian sodium channel function are available.
- Several cDNAs encoding α-scorpion neurotoxins were cloned by us and their sequences determined. Furthermore, we established a reliable system for *ex vivo* functional expression of LqhαIT (Zilberberg *et al.*, 1996) which is most suitable for site-directed modificational analysis and determination of its three-dimensional structure.
- Alpha scorpion toxins display different binding capabilities to various sodium channels of the same organism or in different animals. For example, AaH2 and Bom3 (*Buthus occitanus mardochei*) do not displace each other from the receptor site in rat brain synaptosomes (Vargas *et al.*, 1987); Lqq4 hardly affects insects when compared to the strong anti-insect toxicity of LqhαIT (Zilberberg *et al.*, 1996); the anti-insect/mammal toxicity ratio can be altered (Zilberberg *et al.*, 1996). These data suggest that the binding ratio of alpha toxins to various channels could be modified by genetic means.
- The three-dimensional structure of three alpha scorpion toxins, AaH2, AaH3 and BeM9 (*Buthus eupeus*), has been solved by X-ray crystallography and ^{1}H 2D-NMR (Fontecilla-Camps *et al.*, 1982, 1988; Mikou *et al.*, 1992; Pashkov *et al.*, 1988). This enables inclusion of assayable predictions in the genetic approach based on a combination of comparative analyses of alpha toxin sequences and three-dimensional structural models.
- LqhαIT-cDNA was placed under a strong promoter (polyhedrin) in the genome of the insect pathogen *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) and viral isolates with enhanced insecticidal properties were obtained (Chejanovsky *et al.*, 1995).

2. Structure–activity relationship of alpha scorpion toxins

Several approaches have been undertaken in studying structure–function relationship of scorpion toxins: chemical modifications introduced to isolated toxins (Darbon *et al.*, 1983; El Ayeb *et al.*, 1986a; Kharrat *et al.*, 1989); immunochemical studies to identify regions participating in binding to the receptor site in sodium channels (El Ayeb *et al.*, 1986b); a biophysical approach to determine the three-dimensional structure (Fontecilla-Camps *et al.*, 1982, 1988; Mikou *et al.*, 1992; Pashkov *et al.*, 1988); and a genetic approach using isolated cDNA clones and *ex vivo* expression systems (Bougis *et al.*, 1989; Chejanovsky *et al.*, 1995; Gurevitz *et al.*, 1991; Maeda *et al.*, 1991; Martin-Eauclaire *et al.*, 1994; Stewart *et al.*, 1991; Zilberberg and Gurevitz, 1993; Zilberberg *et al.*, 1996).

2.1 Chemical modifications

A number of chemical modifications of various toxins have been carried out for localizing residues involved in the binding to the receptor or in their pharmacological properties. Biotinylation of Lys56 in AaH1 and Lys58 in AaH2

of *A. australis* Hector or in Lqq5 of *L. q. quinquestriatus* reduced the toxicity by 80–99% (Darbon *et al.*, 1983). Arg2 and Arg60 were modified in the toxin AaH1 by *p*-hydroxyphenylglyoxal causing a reduction of 60–70% in toxicity (Kharrat *et al.*, 1989). Arg2 modified by 1,2-cyclohexanedione in AaH3 reduced the binding to the receptor site by 99% (Kharrat *et al.*, 1989). Similar treatment of Lys60 in Lqq5 caused 40% loss of activity (Darbon *et al.*, 1983). Nitration of Tyr residues in AaH3 by tetranitromethane caused reduction of 50% in the activity when either Tyr14 or Tyr60 were affected. Nitration of both residues reduced the activity by 68%. When Tyr5, Tyr14 and Tyr60 were nitrated, the remaining activity was 10% (Kharrat *et al.*, 1989). Sulphenylation of Trp38 in AaH2 decreased the activity to 9% (Kharrat *et al.*, 1989). The same treatment of Trp45 in AaH3 did not affect the toxicity (Kharrat *et al.*, 1989). Proteolytic cleavage of the N-terminal pentapeptide of Bom3 of *Buthus occitanus mardochei*, reduced its activity to 10% (El Ayeb *et al.*, 1986a). All of these residues are located in or near a conserved hydrophobic region proposed by Fontecilla-Camps *et al.* (1982) to carry the 'toxic site'. A conclusion drawn out of these studies was that all important residues are clustered on one face of the α anti-mammal toxin, suggesting a multipoint interaction with residues of the mammalian sodium channel.

2.2 *Immunochemical studies*

An immunochemical approach using five antibody populations specific towards various regions of the toxin AaH2, was conducted to probe the interaction of the toxin with its receptor site. The results revealed two antigenic sites around the disulphide bridge 12–63 and one encompassing residues 50–59 whose blocking avoided binding to the receptor site (El Ayeb *et al.*, 1986b). Monoclonal antibodies that recognized an epitope which included Lys58, Val10 and His64, had similar effects. However, antibodies directed against stretches 19–28 and 28–39 did not affect the capability of the toxin to bind to its receptor site.

2.3 *Determination of three-dimensional structure*

The three-dimensional structures of the α-anti mammal toxins AaH2 and AaH3 from *Androctonus australis* Hector (Fontecilla-Camps *et al.*, 1982, 1988; Mikou *et al.*, 1992), and BeM9, an α-anti mammal toxin from *Buthus eupeus* (Pashkov *et al.*, 1988), have been determined by X-ray crystallography and ^{1}H 2D NMR. These data confirmed the hypothesis that these toxins share a very similar carbon backbone structure. This fact is important when structure–activity relationship of such a toxin is being studied by using a comparative approach.

2.4 *Genetic approach*

Several cDNA clones encoding scorpion neurotoxins have been cloned and in some cases expressed either in COS cells (Bougis *et al.*, 1989) or insect cultured cells

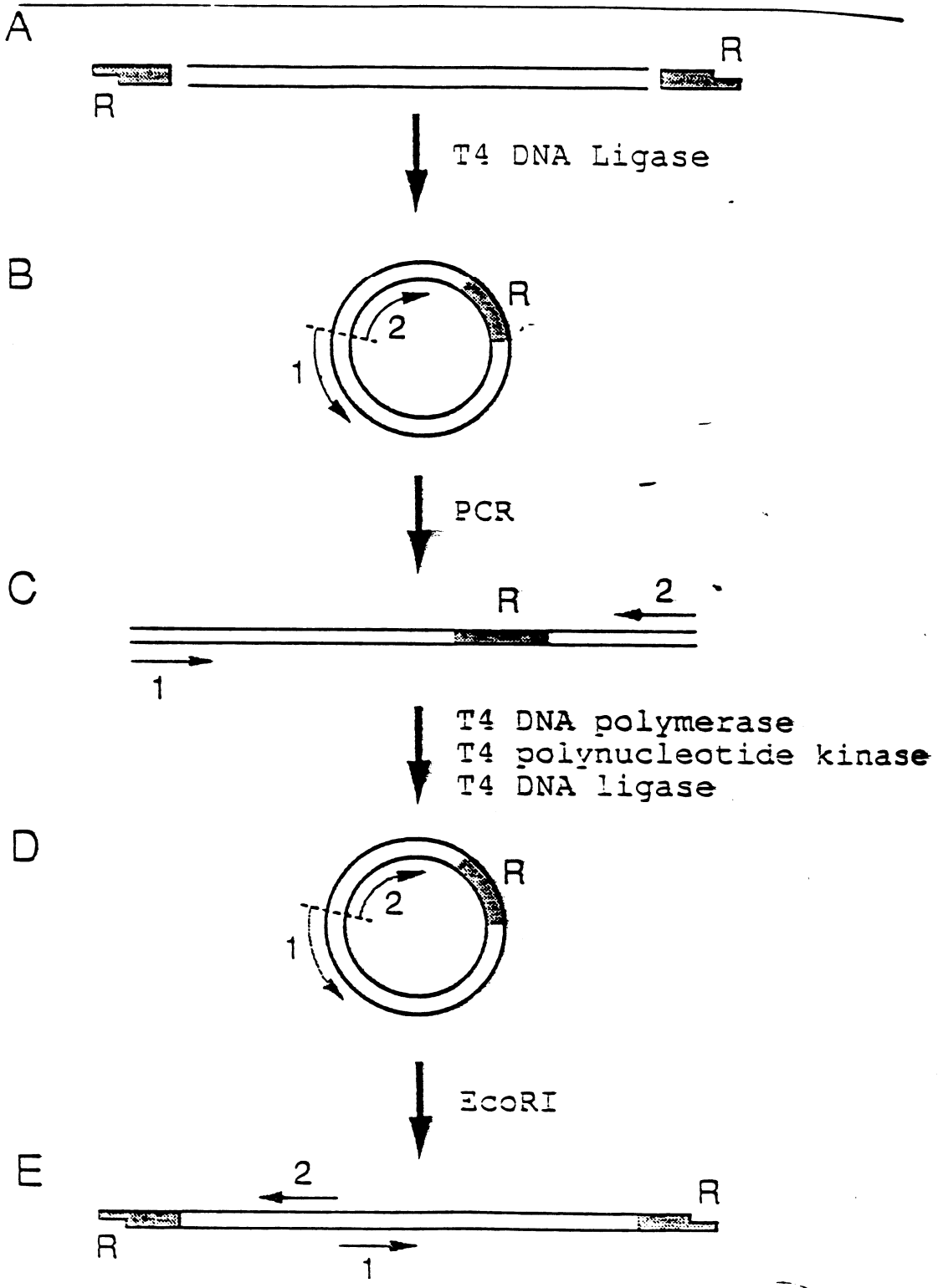

Figure 2. Isolation of cDNA clones. A, molecular adaptors containing unique restriction sites, are ligated to the cDNA mixture; B, circularisation of the cDNA by T4 DNA ligase followed by inverse-PCR (the 'back to back' primers are indicated); C, the PCR product (note the central position of the adaptor and former positions of the primers); D, the linear PCR product is circularised by T4 DNA ligase after 'fill-in' and 5'-phosphorylation; E, digestion with EcoRI generates a linear clonable PCR-product. R indicates EcoRI site. 1 and 2 designate primers.

using a baculovirus vector (Chejanovsky *et al.*, 1995; Maeda *et al.*, 1991; Stewart *et al.*, 1991). However, genetic modifications using these expression systems have not been reported. We have established an expression system that is most suitable for genetic alteration studies. We first isolated (Figure 2) and characterized several cDNAs encoding different alpha toxins from the Israeli yellow scorpion (*L. q. hebraeus*) (Gurevitz *et al.*, 1991; Zilberberg and Gurevitz, 1993). The cDNA clone of LqhαIT, a unique alpha toxin with a high ratio of anti-insect/mammal activity, was successfully expressed in *E. coli* by using the pET-11c translational vector bearing a strong T7 promoter (Figure 3). The toxin obtained in large insoluble amounts was completely denatured and then reoxidized under controlled conditions (Zilberberg *et al.*, 1996). The recombinant protein was purified by HPLC and analysed for its activity. Toxicity was estimated by injection to blowfly larvae and mice; electrophysiological properties were determined by current and voltage clamp experiments using a cockroach isolated axon, and binding to the receptor was performed with a neuronal membrane preparation from locust synaptosomes. The recombinant toxin, engineered with an additional methionine residue at its N-terminus, was found to be identical to the authentic toxin purified from the scorpion venom (Eitan *et al.*, 1990). This expression system was used for amino acid substitutions of specific C-terminal and N-terminal residues. Design of the modifications was based on comparative analyses with other alpha toxins of high anti-mammal/insect toxicity ratio (Lqq4 and AaH2; Figure 1). The substitutions of three residues on the C-terminal side had a minute effect on the toxicity to blowfly larvae and on the binding affinity to insect neuronal membranes. However, the ratio of anti-insect/mammal activity (toxicity and binding to the receptor) increased several-fold (Zilberberg *et al.*, 1996). Conversely, the substitution of three amino

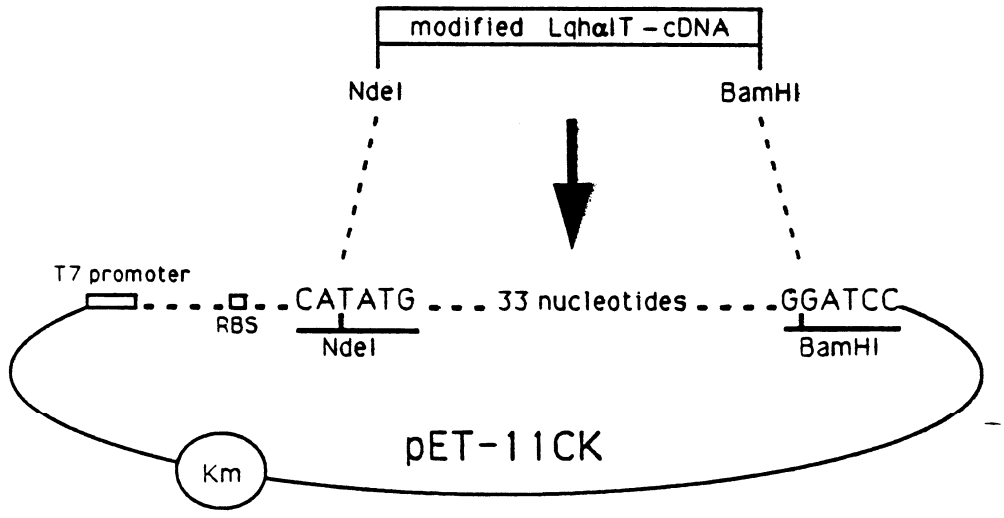

Figure 3. Construction of the expression vector. The engineered cDNA was inserted between the NdeI and BamHI sites in the vector pET-11c, under the control of the T7 promoter.

Table 1. Biological activity of variant genetically modified LqhαIT toxins.

Toxins and modificants	ED_{50} (ng 100 mg^{-1} body weight)	IC_{50} (nM)	LD_{50} (ng g^{-1} body weight)
LqhαIT-rec	14	0.38 ± 0.1	250
C-terminus	20	0.44 ± 0.1	1 000
N-terminus	3000	2500 ± 1500	70 000
Lqq4	950	Unknown	70

ED_{50}, Effective dose 50% determined on 100–150 mg blowfly larvae.
IC_{50}, Inhibition concentration 50% determined on locust synaptosomal membranes.
LD_{50}, Lethal dose 50% determined on 20 g ICR mice.
LqhαIT-rec stands for the nonmodified recombinant toxin.
C-terminus and N-terminus stand for the modified toxins.
Lqq4, purified toxin from the venom of *L. q. quinquestriatus*.

acid residues on the N-terminal side of the toxin, reduced the biological activity (ED_{50} and IC_{50}) by several orders of magnitude (Table 1). Thus, the *E. coli* expression system followed by reconstitution of active toxins *in vitro* paved the way for a genetic study aiming to clarify the toxic site of scorpion alpha nuerotoxins and the molecular basis for the 'animal group specificity' phenomenon.

3. Baculovirus-mediated targeting of scorpion toxins

The nervous system of insects serves as a major target for conventional efforts of insect control which relies currently on hazardous chemicals of four groups: organochlorines, organophosphates, carbamates and pyrethroids (Elliot, 1986). It is our aim to develop new safer means for pest control by utilizing natural anti-insect agents. Insecticidal polypeptidic neurotoxins from scorpion venoms targeted to insect sodium channels of excitable tissues can be used for this purpose. However, in order to be efficiently utilized, they require a delivery system to reach their target sites. In this respect, an insect pathogen which naturally infects and spreads within the insect host and is amenable for genetic engineering, should be an ideal vector.

Baculoviruses, double stranded DNA viruses, infect insects and do not infect mammals (Huber, 1986). More than 500 baculovirus species have been isolated from various insect orders (Martignoni and Iwai, 1986; Matthews, 1982) and almost all serious lepidopterous pest species are susceptible to infection by one or more species of baculovirus. The baculoviruses are divided into three groups: the non-occluded viruses (NOVs) characterized by the absence of an occlusion body; the granulosis viruses (GVs) comprised of a single virion occluded in a polyhedral capsule; and the nuclear polyhedrosis viruses (NPVs) in which many virions are occluded in a polyhedral crystal. The polyhedra increases the stability of the encapsulated viruses, protects the viral particles from environmental effects, and its ingestion by insect larvae initiates the infectious cycle. The polyhedra dissolves in the alkaline conditions of the insect midgut and the released virions fuse with

epithelial cells. The virions migrate through the cytoplasm, uncoat in the nucleus (Granados and Lawler, 1981), replicate and then bud through the plasma membrane. This process results in a secondary infection of various tissues of the larval host. It was recently shown that the major pathway for spread of the viral infection is through the tracheal system (Engelhard *et al.*, 1994). At a later stage of infection, occlusion bodies containing mature virions are produced in the infected cell nuclei. The continuous replication of the virus results in the death of the infected larvae. The cadavers full of polyhedra rupture easily and millions of polyhedra are released to the surrounding foliage and soil.

There are several advantages in utilizing baculoviruses as biopesticides:

- They infect only arthropods and are not pathogenic to vertebrates or plants (Carbonell and Miller, 1987; Huber, 1986; Miller, 1988; Miller *et al.*, 1983; Summers *et al.*, 1975).
- Most baculoviruses are specific to a single family or genus of insects (Entwistle and Evans, 1985).
- No undesirable residues are left in the environment after baculovirus use (Huber, 1986).
- They are used successfully with other control agents in integrated insect pest management (IPM) systems (Huber, 1986).
- Eukaryotic proteins expressed by recombinant baculoviruses in insects are properly processed posttranslationally (glycosylation, phosphorylation, amidation and internal cleavage of polypeptides), accumulate to high amounts and are biologically active.

Baculoviruses have been registered in the past as bioinsecticides despite a major disadvantage in their slow killing rates (6–10 days post-infection) of insect pest larvae. During this period of time, extensive damage to the crop may occur (Huber, 1986). This problem illustrates the need to engineer baculoviruses with foreign genes encoding insecticidal agents which impair normal functions in the insect development, metabolism and neural system. The availability of insect cell cultures, used for the engineering of recombinant baculoviruses (for a comprehensive review see Luckow and Summers, 1988), has motivated us and others (Chejanovsky *et al.*, 1995; Hammock *et al.*, 1990; Maeda *et al.*, 1991; Stewart *et al.*, 1991; Tomalski and Miller, 1991) to utilize baculoviruses as suitable vectors for the expression of various anti-insect components in insect pests.

3.1 *Recombinant baculoviruses expressing polypeptides involved in insect development and metabolism*

Insect neuropeptides and hormones have been expressed by using baculoviral vectors (Bonning and Hammock, 1992; Hammock *et al.*, 1990; Maeda, 1989). However, only the *Bombyx mori* virus (BmNPV) expressing the diuretic hormone from *Manduca sexta* revealed an increased insecticidal effect on *Bombyx mori* larvae by 20% reduction in time to kill as compared with the wild-type virus (Bonning and

Hammock, 1992). The first insect enzyme expressed via a baculovirus vector was the juvenile hormone esterase (JHE), responsible for degradation of the juvenile hormone (Hammock *et al.*, 1990). The recombinant enzyme reduced the amount of diet consumed by first instar *Trichoplusia ni* but no effect was observed in the other instar stages. Two mutant forms of JHE, constructed for improved stability, were found to have an improved effect of reducing the time to kill by 30% when compared to the wild-type baculovirus (Bonning and Hammock, 1992). Another approach was undertaken by O'Reilly and Miller (1991) by deleting the gene encoding ecdysteroid UDP glucosyl transferase (EGT) from the genome of the baculovirus AcNPV (this enzyme blocks the activity of the moulting hormone ecdysone). Viruses lacking the gene coding for EGT reduced by 20% the time to kill *Spodoptera frugiperda* when compared to wild-type.

3.2 *Recombinant baculoviruses expressing toxin genes* –

The expression of a number of different toxins in *Spodoptera frugiperda* cells (SF9 and SF21 cell lines) has been obtained by using the baculovirus vector AcNPV. Among them, BeIT, an anti-insect toxin from the scorpion *Buthus eupeus* (Carbonell *et al.*, 1988), δ-endotoxin from the bacterium *Bacillus thuringiensis* (Merryweather *et al.*, 1990), TxP1 from the mite *Pyemotes tricini* (Tomalski and Miller, 1991) and AaIT from the scorpion *Androctonus australis* (Maeda *et al.*, 1991; McCutchen *et al.*, 1991; Stewart *et al.*, 1991). TxP1 and AaIT reduced the time to kill *Heliothis virescens* larvae by 30% as compared to the wild-type virus. Larvae infected by recombinant baculoviruses expressing the AaIT toxin fell off the plant 5–8 hours before death and were unable to feed or recover (Hoover *et al.*, 1995; Hoover *et al.*, in preparation; Table 2). This resulted in 60% reduction in damage to the plants as compared to larvae infected by the wild-type virus (Figure 4). This result demonstrates that lethal times (LTs) do not necessarily represent the ultimate

Table 2. Oral infectivity of wild-type and recombinant AcNPV-AaIT to *Heliothis virescens* larvae.

Time post infection (h)	Mortality (%)	
	Wild-type	Recombinant
24	0	0
48	0	0
72	0	26
96	7	72
120	20	94
144	50	100
168	72	100
192	90	100

Three replicates (24 larvae each) of third instar *Heliothis virescens* larvae were fed with virus contaminated diet (4000 PIB/plug). Mortality was recorded daily and the data were analysed using a probit analysis program (Russell *et al.*, 1977). LT_{50} = 82 hours post-infection.

Figure 4. Reduction in feeding damage by *Heliothis virescens* larvae treated with recombinant and wild-type baculoviruses. Tomato plants were infected with third instar *H. virescens* larvae. Prior to the infection, the larvae were treated with wild-type (represented by the plant on the left) or recombinant AcNPV-AaIT (represented by the plant on the right).

parameter for evaluating repelling efficacy of pest larvae because feeding stops earlier and crop damage is then minimized.

We engineered a recombinant AcNPV with the LqhαIT-cDNA (Chejanovsky *et al.*, 1995) under the control of the potent polyhedrin promoter (Smith *et al.*, 1983) and obtained expression of the toxin in *Spodoptera frugiperda* culture cells. The functional LqhαIT produced was processed (leader peptide removed) and secreted by the cells. Toxicity of the recombinant toxin was demonstrated on blowfly (*Sarcophaga falculata*) and on lepidopterous (*Spodoptera littoralis*) larvae. The bioinsecticidal effect of the recombinant baculovirus was assayed on *Heliothis armigera* larvae. A significant reduction in the time of killing and in the mean weight gained by the *H. armigera* larvae was obtained when compared to wild-type viruses (Table 3).

4. Conclusions and future prospects

The potency and pharmacological specificity of the anti-insect neurotoxins prompt us to clarify their structure–function relationship and toxin–receptor interactions at the molecular level. The fact that the selective neurotoxin interacts with a well conserved membranal structure serving a defined neuronal function, enables its utilization as a specific marker of the above structure and a key for clarifying its function. As such, the anti-insect specific scorpion toxins and their modificants serve

Table 3. Oral infectivity of wild-type and recombinant AcNPV-LqhαIT to *Heliothis armigera* larvae.

Time post infection (h)	Mortality (%)		Weight gain (mg)	
	Wild-type	Recombinant	Wild-type	Recombinant
24	0	0	9.5	7.8
48	0	0	13.1	12.0
72	0	4.5	63.2	36.1
96	11.4	72.7	125.0	43.0

Second instar larvae were fed with virus-contaminated diet (4000 PIB/plug). Mortality and weight gain were determined in population samples of 48 larvae per treatment.

as valuable tools in comparative neurochemistry, neuropharmacology, and developmental neurobiology. The genetic modificational approach may clarify structural elements involved in the 'toxic site' of some toxins which could be further utilized to design non-peptide agents active specifically against insect pests (Gurevitz and Zilberberg, 1994). The results of the chemical, immunochemical and genetic approaches in studying alpha scorpion toxins, can be interpreted according to the three-dimensional structure published for AaH2 and our three-dimensional model of LqhαIT. The 'conserved hydrophobic surface' (CHS), suggested previously to be involved in binding to the receptor site, is in fact located away from the putative binding surface. However, the outstanding impact of the substitutions at the N-terminal region of LqhαIT is probably due to a conformational change in the three-dimensional structure and does not necessarily imply their involvement in binding to the receptor site. Thus, the genetic system has become a powerful tool in helping to determine the structural motifs participating in binding and in conferring insecticidal activity.

The ability to design molecules capable of distinguishing between insect and mammalian nerves is of practical value. The industrial and agricultural significance of the insect-selective peptide neurotoxins can be appreciated on the background of recent advances in developing baculovirus vectors bearing scorpion toxin genes for plant protection (Chejanovsky *et al.*, 1995; Maeda *et al.*, 1991; Stewart *et al.*, 1991; Tomalski and Miller, 1991).

Several approaches can be envisaged to improve further the insecticidal properties of recombinant baculoviruses. The first is by purification of novel proteins with high insecticidal activity from natural venomous parasitoids or predators of lepidopterous larvae, or by generating recombinant toxins with increased potency via genetic alterations. The second is by improving the efficacy of recombinant baculoviruses through the utilization of various promoters, the expression of more than one toxin by each virus, and their combinations. Early expressing promoters would produce most likely smaller amounts of toxin; however, in our experiments we realized that minute amounts of toxin might already be sufficient for the desired effects. Furthermore, stabilization of RNA transcripts or their translated products by genetic means and the

utilization of other leader sequences for better secretion, could improve the efficacy of the viruses. A third approach could use classical selection. Virus isolates from cultured cells that express higher amounts of proteins or replicate at increased rates, could be selected. The insecticidal properties of existing viruses expressing anti-insect selective neurotoxins can also be improved. Injecting different combinations of scorpion neurotoxins revealed a 5–10 fold increase in the toxicity of some combinations used. These results indicate that baculovirus-mediated simultaneous expression of two different toxins would produce an improved selective biopesticide (Herrmann *et al.*, 1995). Another improvement can be achieved by the combined application of toxins with classical insecticidal chemicals leading to a synergistic effect. The combination of a recombinant AcNPV expressing AaIT with very low doses of classical insecticides (LC_{10}) reduced the time for kill by 30–40% as compared to the recombinant baculoviruses alone (McCutchen *et al.*, 1996).

In summary, genetically engineered viral biopesticides have considerable potential for pest control in the future. Their efficacy as compared with classical chemicals, currently under massive use, depends on their efficiency, speed of kill and reduction in crop damage, safety (selectivity and biodegradativity) and on the environmental risks to other organisms (limited host range and transferability among viral species). Extensive studies are presently underway on aspects of safety. For instance, an ongoing project at the Oxford NERC laboratory is evaluating the effects of engineered viruses on non-target lepidoptera. Our laboratory is examining the interaction of the recombinant viruses with beneficial arthropods. Predators of Lepidoptera, the green lacewing *Chrysopa carnea* and the insidious flower bug *Orius insidious* were not adversely affected by feeding on larvae of *H. virescens* infected with recombinant viruses. In addition, a pollinator, the honey bee *Apis mellifera*, showed no significant differences in survival rates when injected with budded virions of wild-type or recombinant baculoviruses (Heinz *et al.*, 1995). More studies and examination of recombinant baculoviruses and their utilization are required to estimate their future potential in replacing hazardous existing insecticides and prior to their registration and commercial application.

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