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# Insecticidal effects of *Buthus occitanus tunetanus* BotIT6 toxin expressed in *Escherichia coli* and baculovirus/insect cells

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## Abstract

BotIT6 is a neurotoxin polypeptide derived from the venom of the scorpion *Buthus occitanus tunetanus* (Bot). Its mature form is composed of 62 amino acids. BotIT6 has been reported to be the most potent toxin from Bot venom that has a strict selectivity for insects. Such toxin may have potential as a potent animal-harmless tool against insects. Using RT-PCR, we isolated and sequenced a cDNA encoding 62 amino acid residues corresponding to the known amino acid sequence of BotIT6. We have expressed a recombinant active form of BotIT6 in significantly high amounts in *Escherichia coli*. We have also engineered the cDNA into the *Autographa californica* Nuclear Polyhedrosis Virus (AcMNPV) genome and expressed the protein under control of the polyhedrin promoter. Supernatants of AcIT6-virus infected Sf9 insect cells exhibit a typical intoxication effect when injected to *Spodoptera littoralis* larvae. Moreover, injection of the recombinant virus showed enhanced insecticidal potency against *S. littoralis* larvae compared with wild type AcMNPV.

**Keywords:** Scorpion; Recombinant toxin; Recombinant baculovirus; Insecticide; Toxicity

## 1. Introduction

Scorpion stings are mainly directed against arthropods and, specifically, soft-bodied insects that serve as prey food. Hence, the selective pressure in scorpion venom may lead to the selection of efficient insect toxins such as excitatory contractive toxins and flaccid depressant toxins (Borchani et al., 1996, 1997; Gurevitz et al., 1998), known as anti-insect toxins (Moskowitz et al., 1998; Froy et al., 1999; Gurevitz et al., 2001; Gordon et al., 2003). They are usually composed of 60–70 amino acids with four pairs of cross-linked disulfide bonds and possess high affinity to sodium channels (Zlotkin et al., 1991; Possani et al., 1999).

When injected into blow fly larvae, the excitatory contractive toxin induces an immediate fast paralysis by depolarizing membrane potentials through a shift in the sodium activation mechanism (Froy et al., 1999). On the other hand, flaccid depressant toxins cause a slow progressive flaccid paralysis and complete relaxation of the body musculature by suppressing the sodium current as well as depolarizing the axonal membrane potential.

Bioinsecticides and insect-resistant genetically modified crops represent possible alternatives to chemical insecticides. Over the past few years, much progress has been made by using *Bacillus thuringiensis* toxin (Peferoen, 1997; Lynch et al., 1999) and proteinase inhibitors (Klopfenstein et al., 1997; Pernas et al., 1998; De Leo et al., 2002) to produce insect-resistant crops through gene transfer and plant regeneration. The potential of these genes however is limited since a high concentration of the gene product is

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required to achieve good protection against insect pests (Estruch et al., 1997), and insects develop resistance (Zlotkin, 1999). The search for new approaches or new genes is a major goal of genetic engineering (Evelyn, 1998), to delay the onset of resistance.

Baculoviruses constitute widely used agents for the bio-control of lepidopteran pests. Their main attractiveness is their selectivity and the absence of pollution (Huber, 1986). However, one of the handicaps limiting the generalization of their use is their relatively low speed of action. To increase this speed of action, recombinant baculoviruses have been constructed that express scorpion, mite and spider neurotoxins under control of potent viral promoters (e.g. polyhedrin, *polh* and *p10* gene promoters). These modifications resulted in a 30–45% reduction in effective time to paralysis (ET50) (Stewart et al., 1991; Gershburg et al., 1998; Regev et al., 2003; Szolajska et al., 2004). Thus, insecticidal efficacy could be improved, but it still requires further modifications to reach the standards set by chemical insecticides such as pyrethroids (Hammock, 1998).

Gershburg et al. (1998) constructed a recombinant *Autographa californica* Nuclear Polyhedrosis Virus (AcMNPV) that expresses the depressant insect toxin LqhIT2. They obtain better results (measured as effective time 50, ET50) than when using excitatory toxin LqhIT1. The combination of both excitatory (LqhIT1) and depressant (LqhIT2) toxins in the same recombinant virus (Regev et al., 2003) shows even better results.

BotIT6, a recently described depressant insect toxin, purified from *Buthus occitanus tunetanus* (Bot) venom (Mejri et al., 2003), is very active against *Blattella germanica* (the lethal doses 50, LD<sub>50</sub> being 10 ng/100 mg body mass). The calculated net charge of BotIT6 is positive (+3) whereas it is negative for classical depressant toxins. The positive net charge is thought to be associated with its high toxicity (Mejri et al., 2003). BotIT6 appears to be a good candidate for the construction of a baculovirus insecticide apt to immobilize the infected insect.

To further understand the mode of action of BotIT6, we have cloned the gene encoding BotIT6 depressant toxin and succeeded to express in *Escherichia coli* a biologically active recombinant toxin. For further characterization, we have expressed the toxin in eukaryotic cells using the baculovirus expression system under the control of a polyhedrin promoter and explored the biological properties of the recombinant virus vs. the wild type baculovirus.

## 2. Materials and methods

### 2.1. Materials

Restriction enzymes were obtained from Pharmacia Biotech, standard molecular biology reagents were purchased from Sigma and Prolabo.

### 2.2. Telson mRNA purification

Scorpions *Buthus occitanus tunetanus* were killed 2 days after venom extraction to allow toxin producing cells to enter their secretory phase. Tissues of telsons were homogenized and total RNA extracted by means of the Guanidium/hot-phenol method (Maniatis et al., 1982). Poly (A)-rich RNA was purified on an oligo(dT)-cellulose spun column (Pharmacia).

### 2.3. Cell lines and viruses

*Spodoptera frugiperda* Sf9 cells were maintained and propagated in TC-100 medium supplemented with 10% heat-inactivated foetal bovine serum. Cells were infected with baculovirus suspensions at a multiplicity of infection (m.o.i) of 10 plaque forming units per cell (pfu/cell). Virus stocks were titrated by plaque assays.

### 2.4. Cloning BotIT6

To amplify the cDNA sequence encoding BotIT6 toxin, two degenerate oligonucleotides were designed according to the BotIT6 amino acid sequence (Mejri et al., 2003).

(i) A forward primer (BotIT6 for1): 5' GAT GGW TAT CCM AAA CAA AAA AAY GGW TGY AAA TA 3' corresponding to the N-terminal residue (+1 to +12) of BotIT6 peptide sequence (ii) a reverse primer (BotIT6 rev1): 5'ACY TCC RCA TTT RTT YYT TTC GTA CCA CCA TTT 3' corresponding to the C-terminal (residues 52–62) residues of BotIT6 peptide sequence.

First strand cDNA was synthesized. BotIT6 sequence was then amplified by BotIT6for1 and BotIT6rev1 as primers (PCR conditions: 3 min at 94 °C followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min). The PCR product of 200 bp was cloned into a pGEM-T vector (Promega, USA). *E. coli* (JM109) was transformed with the ligated product, and the recombinant clones were selected on a Luria-Bertani/ampicillin (100 µg/mL) plate supplemented with Thio-β-D Galactoside (IPTG) and X-Gal according to the method described previously (Sambrook et al., 1989). The plasmid isolated from the recombinant clones was then sequenced using the chain termination method (ABI PRISM DNA sequencer) and M13/pUC forward and reverse primers. The DNA sequences and deduced amino acid sequences were analysed using Clustal V and Mac Molly program.

### 2.5. Expression of BotIT6 in *E. coli*

Two primers (Forward primer (BotIT6for2) 5'-GGAGC GGTACC C ATC GAA CGT GGT GAT GGT TAT CCC AAA-3', and reverse primer (BotIT6rev2) 5'-GCGGCAA GGATCC TTA ACG TCC GCA TTT GTT TTC GTA-3') were designed to amplify the full coding sequence corresponding to mature BotIT6 protein. The forward primer introduced a KpnI restriction site and

site specific protease factor Xa (FXa) (Benkhadir et al., 2004), allowing an insertion in frame with the sequence encoding two IgG binding domains ZZ of *Staphylococcus* protein of pEZZ-18 vector. The reverse primer was designed to introduce the BamHI restriction site and a stop codon TAA just after the R62 residue. The final construct pEZZ-BotIT6 was introduced into *E. coli* HB101.

#### 2.6. Production and purification of fusion protein ZZ-BotIT6

The full-length cDNA of BotIT6 was expressed in the periplasm of *E. coli* as a fusion protein with two ZZ domains of *Staphylococcus* protein A. *E. coli* cells harbouring pEZZ-BotIT6 were grown at different temperatures (28, 30, 37 °C) in LB medium supplemented with 200 µg/mL ampicilline. Cells were harvested and resuspended in lysis buffer (30 mM Tris-HCl, 10 mM EDTA, 20% sucrose, pH 8, 0.5 mM PMSF, 10 µg/mL lysozymes). The lysate was maintained at 4 °C for 2 h. After centrifugation for 15 min at 10,000 rpm, the supernatant was passed through an IgG Sepharose column (Pharmacia). The column was washed with TST buffer (50 mM phosphate buffer pH 7.6, 150 mM NaCl, 0.05% Tween 20) until the OD 280 nm reached the baseline. The gel was washed with 2 volumes of 5 mM ammonium acetate buffer, pH 5. The hybrid proteins were then eluted with 0.5 M acetic acid, pH 3.4 and immediately lyophilized. Purity of the hybrid proteins was checked by SDS-polyacrylamide gel electrophoresis.

#### 2.7. Immunoblot analysis

The fusion proteins were resolved by SDS-PAGE on a 20% polyacrylamide gel. After electrophoresis, gels were stained with Coomassie blue G-250 for identification of protein bands or transferred to polyvinylidene difluoride membrane, then incubated with total rabbit anti-sera raised against the native venom toxin as a primary antibody and after extensive washing with an anti-rabbit IgG conjugated to alkaline phosphatase. Diaminobenzidine (DAB) was used as the substrate to reveal the immunoreactive bands.

#### 2.8. Processing of fusion proteins with factor Xa and characterization of recombinant toxins

Conditions for an efficient cleavage of fusion proteins of ZZ-BotIT6 with FXa (10 mU of protease per microgram of protein substrate) in TBS buffer was determined by analysis of the effect of different temperatures (30, 37, 40 °C), pH (between 6 to 10), protein concentrations (1 mg/mL) and incubation time. The reaction products were analysed by 20% SDS-PAGE under reducing conditions. The rBotIT6 released from the fusion proteins was then

purified by high performance liquid chromatography on C8 columns.

#### 2.9. Construction of recombinant viruses

The BotIT6 cDNA was amplified using the two following primers: (BotIT6for3): 5'-GCG GAG GTT AAC GCT GAT GGT TAT CCC AAA CAA-3' and (BotIT6rev3): 5'-GCG CCA AGA TCT TTA ACG TCC GCA TTT GTT GGT-3'. The forward primer introduced (i) a HpaI (underlined) restriction site (ii) the three last codons of the AcMNPV EGT signal peptide sequence and (iii) the six first codons of BotIT6. The reverse primer introduced a BglII site (underlined), a stop codon (bold-face) and the six last codons of BotIT6. The PCR product was then digested by HpaI and BglII and purified. The HpaI-BglII fragment of 200 bp containing the BotIT6 sequence and 3 codons of EGT signal peptide was isolated and introduced into the HpaI-BglII sites of pUC-PS EGT (Marchal et al., 2001) carrying the sequence encoding the N-terminal part of the EGT signal sequence, allowing the fusion in frame of the BotIT6 sequence with the signal peptide sequence. The reconstituted sequence was isolated after digestion with BamHI and BglII and inserted downstream of the polyhedrin promoter into the pGmAc116T baculovirus transfer vector (Poul et al., 1995) (Fig. 4) giving the construct pGmAc116T-IT6. Sf9 cells were transfected with 500 ng of purified viral DNA and 5 µg of pGmAc116T-IT6 plasmid DNA using lipofection (DOTAP, Roche, Germany). The screening and purification of the recombinant baculoviruses (AcIT6) were carried out by plaque assays as described in Summers and Smith (1987). The genome of the recombinant virus expressing BotIT6 toxin was controlled by Southern blot (Southern, 1975) using a BotIT6 specific probe.

#### 2.10. Protein expression

Sf9 cells were infected at an m.o.i. of 10 pfu/cell. After 4 days incubation at 28 °C, cells were harvested and lysed after several cycles of freezing and thawing. The total cell extract was analysed for the presence of BotIT6 by 20% SDS-PAGE, by western blot with anti-LqqIT2 rabbit serum (Cestèle et al., 1997) (1000 fold dilution).

#### 2.11. Toxicity assays

rBotIT6 expressed in *E. coli* purified on HPLC was lyophilized. Distilled water was added just before experimentation and protein quantification. The toxicity of purified protein was tested on the male cockroach *B. germanica* (40 mg body weight) anaesthetized by CO<sub>2</sub>. At least eight *B. germanica* were used per dose (8-12). A volume of 1 or 2 µL of various concentrations of the diluted toxin was injected into the intersegmental membrane of the lateral side of the abdomen, and the needle was kept in place for a few minutes. Insects were observed

during 2 h post-injection, and toxicity was monitored by a lethality test each 24 h for 4 days. To avoid experimental differences with previous experiments (Mejri et al., 2003) between insect strains and protocol assays, the effect of rBotIT6 was compared with toxicity obtained with crude venom, and purified native BotIT6 (nBotIT6). Used concentrations were 10 and 20, 4–20, 5–60 ng/μL for venom, nBotIT6 and rBotIT6, respectively. Previous experiments on venom and nBotIT6 expressed results as ng/100 mg *B. germanica*, and the insect weight was estimated to be 100–120 mg (Mejri et al., 2003; Borchani et al., 1997).

Toxicity of rBotIT6 expressed in the baculovirus expression system was checked by injecting *S. littoralis* larvae. They were obtained from a laboratory colony reared on semi-artificial diet (Poitout and Bues, 1974) at 22±0.5 °C, 70% humidity, with 16 h photoperiod. Larvae aged 11 days, with average weight (160±3 mg) were utilized. They were injected with 8 μL each of infectious supernatant of either unmodified parental baculovirus AcMNPV (wt), or the recombinant baculovirus expressing BotIT6 toxin (AcIT6).

Preliminary experiments indicated that a dose of 10<sup>5</sup> pfu was sufficient to kill all larvae, thus the two baculoviruses were diluted with PBS to achieve each dose (10<sup>5</sup>, 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup> and 10 pfu) in 8 μL. The control group was injected with 8 μL PBS. To evaluate paralyzing symptoms, groups of 12 larvae were injected with 8 μL of venom, wt type frozen virus and AcIT6 frozen virus.

To measure LT<sub>50</sub> (Time for 50% mortality), a total of 120 larvae were injected with AcIT6 virus supernatants, 120 with the unmodified control virus and 24 with PBS. Survival was scored every 6 h. For each experiment, mean values ± standard deviation (SE) of mortality data were calculated. The cumulative mortality across the assessment period was analysed with Kaplan–Meier survival in SPSS (Statistical Package for Social Sciences in Personal Computers) 8.0 for Windows to determine if differences between the treatments were significant.

### 3. Results

#### 3.1. Cloning, sequencing and expression of BotIT6

##### 3.1.1. Cloning and sequencing

mRNA isolated from frozen scorpion telsons served as a template for the first strand cDNA synthesis with the degenerate primer BotIT6rev1 specific to the C-terminal sequence of BotIT6. PCR amplification of this cDNA was then performed using BotIT6rev1 and BotIT6for1, an N-terminal specific primer.

The PCR fragments of approximately 200 bp were purified and cloned into the pGEM-T vector. The DNA fragments were then purified by a PCR purification kit and cloned into the pGEM-T vector. White colonies were screened by PCR with Universal M13 and specific primers. Positive clones were sequenced. The nucleotide sequence

analysis displayed an open reading frame (ORF) of 180 bp encoding a polypeptide of 62 amino acids (Fig. 1) corresponding to the mature toxin BotIT6.

##### 3.1.2. Production and purification of recombinant BotIT6

To obtain large amount of rBotIT6 toxin, we expressed the BotIT6 in an *E. coli* system as a fusion protein. BotIT6 sequence encoding the mature toxin was inserted in frame with a sequence encoding the signal peptide sequence of protein A, two IgG-binding domains (14 kDa), and an IEGR site specific to protease FXa. The final construct pEZZ-BotIT6 was introduced into HB101 host cells. Recombinant BotIT6 was produced as a major fusion protein. Analysis of a crude extract prepared after osmotic shock revealed that an IgG affinity-purified protein with apparent molecular mass of 21 kDa (Fig. 2) was present. This result is in agreement with immunochemical

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GAT GGT TAT CCC AAA CAA AAA AAC GGA TGC AAG TAT
D   G   Y   P   K   Q   K   N   G   C   K   Y

GAT TGT ATT ATA AAT AAT AAG TGG TGT AAT GGT ATA
D   C   I   I   N   N   K   W   C   N   G   I

TGC AAA ATG CAT GGT GGC TAT TAT GGA TAC TGT TGG
C   K   M   H   G   G   Y   Y   G   Y   C   W

GGC TGG GGA GGT CTT CTT GCC TGT TGG TGC GAA CCG
G   W   G   L   A   C   W   C   E   G   L   P

GAA GAC AAG AAA TGG TGG TAC GAA ACC AAC AAA TGC
E   D   K   K   W   W   Y   E   T   N   K   C

GGA CGT
G   R

```

Fig. 1. Nucleotide sequence and the deduced amino acid sequence of the c-DNA encoding mature BotIT6.

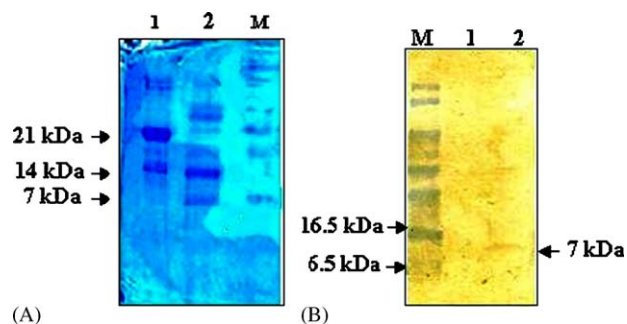


Fig. 2. SDS gel and immunoblot analysis of recombinant BotIT6. (A) Expression of the recombinant fusion protein between BotIT6 and ZZ domain of *Staphylococcus*. SDS-PAGE of purified fusion protein (lane 1); cleavage products purified after digestion with factor Xa (lane 2) and M: Molecular weight standards (kDa). (B) Immunoblot analysis of BotIT6 expression in Sf9 insect cells. Cell extracts prepared at 96 h post-infection were subjected to 20% SDS-PAGE proteins were then transferred into PVDF membranes and immunoblot analysis using anti-LqqIT2 anti-sera. (Lane1): Sf9 cells infected with wild type AcMNPV. (Lane2): Sf9 cells infected with AcIT6. M: Molecular weight marker (Broad range P7708S).

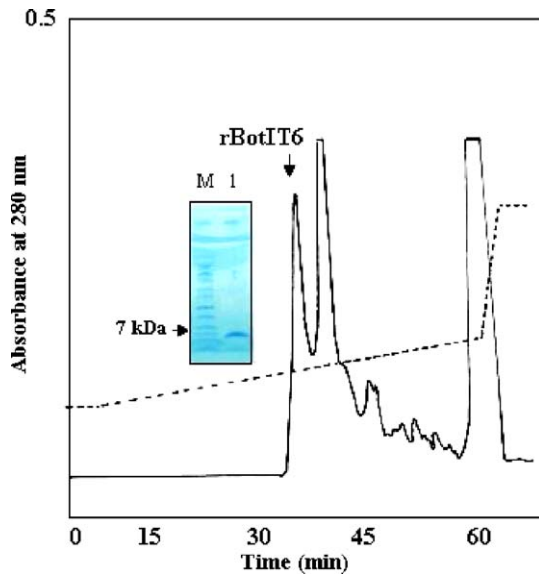


Fig. 3. HPLC purification of recombinant BotIT6 in *E. coli*: The cleavage product of ZZ-BotIT6 with Factor Xa was fractionated by reversed chromatography on C8 column. The column was equilibrated with 20% acetonitrile (0.1% trifluoroacetic acid) and eluted with a linear gradient of 20–40% acetonitrile for 60 min at a flow rate of 0.8 mL/min. Inset: 20% SDS-PAGE purified rBotIT6. Lane 1: Purified rBotIT6 appear as a band of approximately 7 kDa. M: Molecular weight standards (kDa).

recognition of the 21 kDa protein by anti-BotG50 antibodies (data not shown). It represents more than 90% as judged by SDS coomassie blue gels (15% acrylamide). The yield of fusion protein estimated by absorbance at 280 nm, was 3 mg/L.

Optimal cleavage by FXa (10 U/mg) appears to occur at: pH. 8; 37 °C and during 24 h. In these conditions, the fusion protein is cleaved into 14 and 7 kDa polypeptides corresponding to ZZ domains and rBotIT6. The average yield of the cleavage is about 70% of the total hybrid (Fig. 2A).

The rBotIT6 was purified on a C8 reverse phase column HPLC. Toxicity test and co-elution with the native BotIT6 (nBotIT6) were used to identify the peak corresponding to rBotIT6. The peak eluted with (28%) acetonitrile at a retention time of 32 min (Fig. 3) is homogenous and shows a unique band at 7 kDa by SDS-PAGE (Fig. 3 inset). This peak is the only one exhibiting toxicity towards *B. germanica*: it corresponds to recombinant BotIT6 (rBotIT6). Amino acid analysis of the purified product shows amino acid ratios that are consistent with the value deduced from its composition (data not shown).

### 3.1.3. Toxicity of rBotIT6

The biological activity of rBotIT6 was estimated for paralysis and lethality on the cockroach. These effects were compared with those of venom and nBotIT6.

Venom induced paralysis in some insects (leg extension, uncoordinated motion, fall on the dorsal side), and the LD<sub>50</sub> at 24 h was 32.5 ng/100 mg body weight. Symptomatology was the same as for nBotIT6, with LD<sub>50</sub> approxi-

mately 17.5 ng/100 mg, but we observed that paralysis may be reversible, and that in some cases, death occurred without preliminary symptoms. Different samples of HPLC C8 purified batches of rBotIT6 were used. The observed symptomatology was the same as for nBotIT6. General mean for LD<sub>50</sub> 24 h post-injection of rBotIT6 was 30 ng/cockroach or 75 ng/100 mg body weight, corresponding to about 270 pmol/g in our experimental conditions.

### 3.2. Cloning and expression of BotIT6 by AcMNPV/Sf9 insect cells

As the signal peptide for BotIT6 toxin secretion is not known, the 18 amino acid signal sequence of the EGT gene from AcMNPV (M T I L C W L A L L S T L T A V N A) was used. Transfer vector construction was performed after modification by PCR amplification of the cDNA sequence that encodes the mature sequence of the toxin. The plasmid pGmAc116T-IT6 containing an ORF encoding the signal peptide and the BotIT6 (7 kDa) mature sequence was selected by DNA sequencing (Fig. 4). Sf9 insect cells were co-transfected by lipofection with viral DNA (500 ng) and the transfer vector pGmAc116T-IT6 (5 µg). Four isolated clones were picked, and amplified for further studies and characterization of the recombinant virus (AcIT6).

The genome of recombinant viruses were first analysed by PCR using BotIT6for2/BotIT6rev2 primers and for116 (a specific primer hybridizing upstream of the polyhedrin promoter)/BotIT6rev2 primers. Amplification of approximately 200 and 450 bp was observed, respectively (data not shown). One recombinant baculovirus clone was amplified for further genetic characterizations. Both recombinant and parental virus genomes were analysed by Southern blot (data not shown) using BotIT6 specific. A 7240 bp labelled fragment was revealed, indicating the presence of BotIT6 in the polyhedrin locus (Ayres et al., 1994).

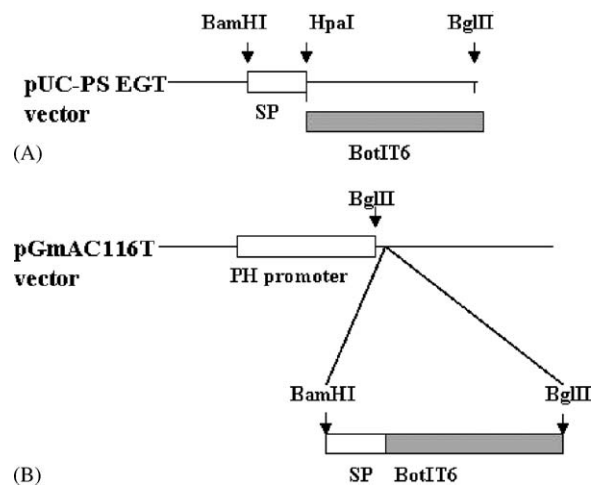


Fig. 4. Gene organization of recombinant baculoviruses bearing toxin gene. (A) EGT signal peptide sequence is inserted in frame with BotIT6 gene. (B) Cloning of BotIT6 gene in pGmAc116T vector downstream of the pH promoter.

Samples of AcIT6-infected cell extracts were analysed by 20% SDS PAGE. The gel was transferred onto PVDF membrane and immunodetection using anti-LqqIT2 antibodies. Detection of the toxin was performed with anti-rabbit IgG antibody conjugated to alkaline phosphatase, using 3 amino, 9 ethyl, carbozol and dimethyl formamide as substrate. In AcIT6-infected cells a protein band of 7 kDa apparent mass was recognized. This product was not observed in cells infected with the parental baculovirus (Fig. 2B).

### 3.3. Toxicity properties of recombinant baculoviruses

To assay the activity of the recombinant toxin secreted into the cell culture medium, the crude supernatant of cells infected with either AcIT6 or the parental (wt) virus were frozen to inactivate the budded virus, then injected into *S. littoralis* larvae (8  $\mu$ L/larvae). The paralysis induced was compared to that caused by the injection of the native scorpion venom (8 ng/larvae). The strongest paralyzing effect was exerted by venom almost immediately post-injection (20 s), but the effect was noted 10 min post-injection with the recombinant virus (AcIT6). No toxicity was observed after injection of wild type frozen virus.

The effect of the expression of rBotIT6 on the virus was analysed by comparing the speed of action of the recombinant AcIT6 to that of the parental virus (wt). Freshly produced budded viruses were titrated by plaque assays, and then diluted to give the appropriate virus inoculums per larvae. They were obtained with high titers ( $10^7$ ;  $2 \times 10^7$  pfu/mL), respectively, for parental and recombinant virus suggesting the absence of toxicity of the rBotIT6 in cultured cells.

Moreover, toxicity studies on freshly obtained viruses resulted in 90% larvae killing by all doses of viruses tested ( $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$  pfu). Larvae injected by the solvent (PBS) alone did not show any killing.

Global analysis of the data by SPSS confirmed that the difference in the killing rate of 50% for the  $10^2$  virus doses was reached at about 5.17 (3.89–6.44) days post-injection by AcIT6 and at 7.75 (6.62–8.88) days post-injection by the parental virus. The difference is statistically significant ( $P < 0.005$ ). This difference in killing speed was mainly shown at  $10^2$ – $10^3$  viral particles (Fig. 5).

## 4. Discussion

BotIT6 is an anti-insect depressant toxin purified from the venom of *Buthus occitanus tunetanus*, and thus it has the potential to be a potent insecticide. The interesting aspect of BotIT6 is its strict insect-selectivity. As shown in the study of Mejrj et al. (2003), this toxin seems to be very active on the insect-VGSC (Voltage Gated Sodium Channel) but does not affect its mammalian counterpart. For this reason, it could serve as a template for further development of new insecticides.

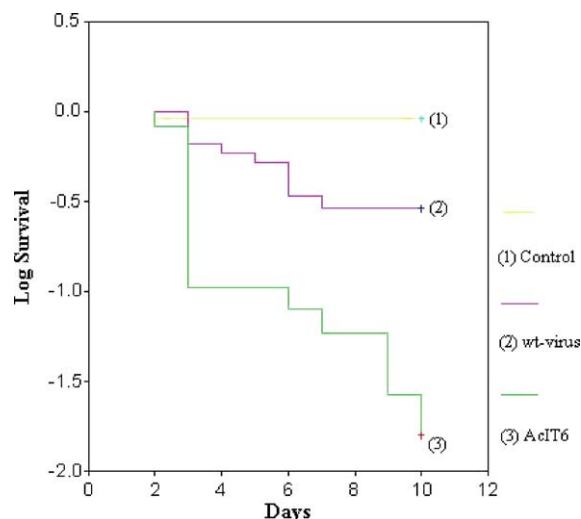


Fig. 5. Cumulative mortality (in percentage) of *Spodoptera littoralis* 24 instar larvae, injected with  $10^2$  pfu of buffer, wt and AcIT6. Curve (1) larvae injected with PBS; curve (2) larvae injected with wild type virus; curve (3) larvae injected with recombinant virus.

In the first part of this work we isolated a cDNA encoding this toxin with RT-PCR amplification. To express BotIT6 in *E. coli*, we considered the difficulties in obtaining functional small polypeptides in the cytosol of *E. coli* due to their degradation and misfolding (Fiordalisi et al., 1991; Marston, 1986). Our strategy was to produce rBotIT6 as a fusion protein to ZZ domains (Nilsson et al., 1987; Bouhaouala-Zahar et al., 1996; Benkhadir et al., 2004). The use of such a system would allow the export of the fusion proteins to the periplasmic area where the machinery needed for the disulfide bond formation is located (Ducancel et al., 1989; Bouhaouala-Zahar et al., 1996; Benkhadir et al., 2004).

By modifying the culture conditions of *E. coli*, we reached yields of 3 mg/L of ZZ-rBotIT6 fusion protein. After cleavage of the fusion product with FXa, and HPLC purification, 1.1 mg/L of homogeneous soluble active rBotIT6 were obtained. These amounts were sufficient to carry out appropriate biochemical and functional studies.

BotIT6 toxin is produced in natural conditions by eukaryotic cells. We analysed the feasibility of production and secretion of the culture media of the toxin using the baculovirus expression system. To that aim, a recombinant virus was constructed encoding a fusion between a secretion signal originated from a baculovirus gene and the mature form of the toxin.

The cells infected by this recombinant virus, AcIT6, release the mature form of the toxin to the extracellular medium. No visible differences (toxicity) were observed between cells infected by the recombinant virus or by the parental virus. When larvae were injected with supernatants of virus infected cell cultures, a clear toxic effect was observed for AcIT6-infected Sf9 cells, but not for the control. This effect is similar to that observed when

injecting the venom, although the paralysis occurs after a delay. The difference might be due to the presence in the venom of other components that act synergistically, or to higher concentrations of BotIT6 toxin. The mortality observed cannot be due to the virus infection, as the virus particles were inactivated.

A major increase in agricultural productivity has resulted from pest control by chemical insecticides directed mainly at neuronal sodium channels. However, this approach is threatened by resistance build-up among insects and poisoning of non-targeted organisms. To date, no detailed information exists on the per os toxicity of BotIT6 insect toxin to mammals. Our work, by allowing the production of high quantities of active toxin, opens the way to such toxicity studies.

Insect viruses could be used as platforms for mobilization of anti-insect selective toxins. This approach was used with AcMNPV expressing various toxins (Stewart et al., 1991; Chejanovsky et al., 1995; Gershburg et al., 1998; Regev et al., 2003). Moreover, risk assessment studies on non-target organisms have indicated that their future utilization could be environmentally safe (Hammock, 1998). AaIT has been the focus of these experiments because it was considered as the most potent anti-insect specific scorpion neurotoxin (Zlotkin, 1987). Since excitatory, depressant and alpha toxins bind to distinct receptor sites on sodium channel (Gordon, 1997), the engineering of different baculoviruses can reduce the host resistance build-up. In addition, Regev et al. (2003) used a bio-insecticide expressing in parallel two toxins targeting different pathway. They obtained a significant increase in killing speed compared to single viruses or to double infection.

Toxicity studies showed that the baculovirus engineered to express BotIT6 toxin is a better killer than the parental virus. The gain in time is considerable if we remember that the feeding period of lepidopteron larvae extends up to 10 days. This is shortened with the parental baculovirus injection to about 7.75 days and to 5 days with recombinant BotIT6 baculovirus.

With our recombinant construction AcIT6,  $10^2$  virus/injection was sufficient to observe 90–100% mortality. At a dose of  $10^2$  virus/injection, we observe a great difference between the wild type and recombinant baculovirus. Compared to other results with toxins, respectively AaIT, Lqh $\alpha$ IT, LqhIT1 and LqhIT2 (Stewart et al., 1991; Chejanovsky et al., 1995; Gershburg et al., 1998), we can say that AcIT6 is equally potent on *S. littoralis* larvae.

The biological activity of rBotIT6 and AcIT6 as insecticides was demonstrated in their study by the induction of flaccid paralysis and larval mortality following injection. Doses of recombinant BotIT6 required to induce paralysis and death, 75 ng/100 mg, are in the same range as the LD<sub>50</sub> value of 17.5 ng/100 mg for BotIT6 purified from Bot venom glands and injected into *B. germanica*. In addition we can observe higher toxicity with AcIT6 by comparison with wt baculovirus, using lower doses ( $10^2$  virus/injection) on Lepidoptera larvae.

In conclusion, we succeeded in expressing significant amounts of active rBotIT6 in a prokaryotic expression system. Our results indicated that BotIT6 may be a new and promising insecticide. It may be useful as a replacement for chemical insecticides or other traditional biopesticides.

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