MOLECULAR CLONING AND NUCLEOTIDE SEQUENCE ANALYSIS OF ENCODED ANTI-INSECT TOXIN BOTIT2 FROM THE SCORPION *BUTHUS OCCITANUS TUNETANUS* VENOM

R. BEL HAJ RHOUMA*, H. DKHIL, K. BENKHADIR,
L. BORCHANI, M. EL AYEB & H. KAROUI

Laboratoire des Venins et Toxines, Institut Pasteur de Tunis, B.P 74, 1002 Tunis-Belvedère, Tunisia

* *Corresponding author*

RESUME

Certaines toxines présentes dans les venins de scorpions sont beaucoup plus toxiques pour les insectes que pour les autres classes animales et présentent une affinité élevée pour les canaux Na+ dépendants du potentiel membranaire. Plusieurs toxines anti-insectes ont été purifiées du venin de Buthus occitanus tunetanus. En se basant sur les séquences en acides aminés et en utilisant l'amplification par la technique RACE PCR (Rapid amplification of cDNA ends), on a pu cloner, identifier et séquencer l'ADNc d'une toxine anti-insecte purifiée du venin de Buthus occitanus tunetanus. La séquence nucléotidique déduite code pour une toxine de 60 acides aminés, identique à la toxine anti-insecte BotIT2[1]. BotIT2 est similaire aux toxines anti-insectes contracturantes dans son mode d'action, elle est plutôt homologue aux toxines flasques dans sa structure primaire[1].

Mots clés : *ADNc de toxines de scorpions; toxines anti-insectes de type flasque; toxines anti-insectes type contracturant; clonage; séquençage; expression*

ABSTRACT

Numerous toxins from scorpion venoms are much more toxic to insects than to other animal classes, and possess high affinity to Na+ channels. Many of them active on insects were purified from the venom of Buthus occitanus tunetanus. Using amino acid sequences of BotIT2 and RACE-PCR amplification (Rapid amplification of cDNA ends) technique, we isolated, identified and sequenced the nucleotide sequence from the venom glands of the scorpion Buthus occitanus tunetanus. The cDNA encodes a precursor of an insect toxin of 60 amino acid residues. The deduced nucleotide sequence toxin was identical to the determined amino acid sequence of BotIT2[1]. BotIT2 is more similar to the excitatory toxins in its mode of action and to the depressant toxins in its primary structure[1].

Key words : *cDNA scorpion toxin ; depressant insect toxin ; excitatory insect toxin ; cloning; nucleotide; sequencing; expression*

INTRODUCTION

Scorpion venom is a mixture of a large number of small polypeptides. Venom contains toxins selectively active on mammals or other vertebrates[2] and toxins lethal to either insects or molluscs[3, 4, 5]. Scorpion toxins specific to insects are selectively active on lepidopterous and dipterous insects. Sodium channels serve as specific targets for these toxins[6].

According to the mode of biological action, insect toxins have been classified as excitatory - contractive or flaccid - depressant toxins[7]. Based on binding and electrophysiological studies, insect toxins are classified into four categories:

(1) Excitatory insect toxins which bind to insect synaptosomal membranes independently of voltage and act on activation as mammals β toxins[3, 8].

(2) Depressant insect toxins which compete with excitatory insect toxins and block action potentials primarily by depolarising the axonal membrane and finally by suppressing the sodium cur-

rent[9, 8, 10]. Application of very long voltage pulses reveals that flaccid paralysis toxins must be considered as sodium channel openers rather than blockers[11].

(3) α type insect toxins which induce progressive contractions in blowfly larvae, do not compete with the excitatory toxins, slow down the inactivation of sodium channel and bind to insect synaptosomal fractions in a potential independent manner[4, 12, 5].

(4) The fourth type consists of toxins that are potent to both mammals and insects[13, 14].

Based on the observation of Zlotkin et al (1971)[15] that the venom of *Buthus occitanus tunetanus* is among the most toxic to blowfly larvae (0.05µg/100mg body weight), some peptides have been purified and characterized from the venom of *Buthus occitanus tunetanus* like BotIT1, BotIT4, Bot IT5 [5]; BotIT2[1] and BotIT6[16].

BotIT2 is highly active on insects but less active on mammals[1]. This toxin is distinguishable from other scorpion toxins by (i) its new action on the activation kinetics of the insect sodium channel, BotIT2 specifically acts by inducing a new current with very slow activation/deactivation kinetics due to the transformation of normal fast channels into slow ones[17] (ii) its primary structures[1]. Like depressant toxins and unlike α type toxins, BotIT2 is able to displace iodinated AahIT from its binding sites in insect neuronal membranes[18].

Other toxins which have the same characteristic to BotIT2 named (a depressant insect-selective toxin analogue), like LqhIT5 purified from the venom of *Leiurus quinquestriatus hebraeus*[19]; and AaIT5 from the venom of *Androctonus australis Hector*[20], were described.

Cloning, expression and mutagenesis of this toxins (with no depressant activity) by substitution of amino acids located on a specific exposed area should provide a new light on the structure/function relationship of scorpion toxins.

It is reported by Borchani et al.(1996)[1] that the differential properties between BotIT2 and flaccid toxins may be attributed to evolutive substitution occurring at positions: 6-8, 37, 52 and 55, where GYK, K, R and P for BotIT2 are replaced by KRD/RRD, T, T and S, respectively, for LqqIT2 and LqhIT2 depressant insect-toxin.

Anti-insect scorpion toxins become also of major interest insect neurophysiology and insect pest control, due to their specific target sites[21, 22, 23]. The specific action of this toxin against insects provides possibilities for using it as neuropharmacological tool to study insect neuromuscular processes.

In this work, using specific N-terminal and C-terminal sequence of BotIT2, a couple of primers were synthesised and used for 3'RACE amplification (Rapid amplification of cDNA ends). The cDNA of BotIT2 was successfully cloned from mRNA of venom glands of scorpion *Buthus occitanus tunetanus*, sequenced and expressed as fusion protein.

MATERIALS AND METHODS

1- PURIFICATION OF TELSON mRNA

Buthus occitanus tunetanus scorpions were killed 2 days after venom extraction to allow toxin producing cells (venom glands) to enter into secretory phase. The mRNA was extracted from homogenized venom gland tissues by using a quick prep micro mRNA purification kit (Amersham pharmacia). Isolated mRNA essentially free of DNA and protein contamination, has been used directly for first strand cDNA synthesis.

2- DESIGN AND SYNTHESIS OF OLIGONUCLEOTIDES

The oligonucleotides used for PCR were designed and synthesized according to the specific BotIT2 amino acid sequence. The forward primer was IT2a corresponding to the residues Asp-1 to Ile-12 of BotIT2, and the reverse primer was IT2r corresponding to the residues Lys-51 to Cys-60 of BotIT2.

IT2a: 5' GAT GG(A,T) TAT AT(C,T) AAA GG(A,T) TAT AAA GG(A,T) TGC AAA AT 3'

IT2r: 5' CA(A,T) GT(A,G) TT(A,T) GTT TC(A,G) GGT TTC CA(C,T) C(G,T)T TT 3'

3- 3' RACE AMPLIFICATION AND cDNA CLONING STEPS

First strand cDNA used in polymerase chain reaction (PCR) was synthesized from 1 µg mRNA, and was incubated with IT2r primer (100 pmol) and DEPC treated water in 23 µl total volume for 10 min. at 70°C, then dNTP (10mM), Reverse Transcriptase buffer (5X), DTT (0.1M) and Superscript II Reverse Transcriptase were added and incubated 60 min at 50°C. Finally, RNAse H was added and incubated for

20 min at 37°C. The first strand cDNA synthesized was then amplified by PCR primers IT2a and IT2r and the enzyme Pfu polymerase (Pharmacia Amersham). Samples were incubated at 94°C for 3 min, then subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 55 °C for 1 min, and elongation at 72°C for 1 min. The final cycle was followed by an additional extension step at 72°C for 10 min.

After each PCR amplification, 10 µl of PCR reaction products were analysed by electrophoresis on a 2 % agarose gel in TBE buffer and the bands detected by ethidium bromide staining. The PCR products were purified using PCR purification kit (Qiagen). Then an additional amplification with the Taq polymerase (Pharmacia Amersham) is essential for cloning into pGEM-T. Ligated cDNA were introduced into E-coli JM109 competent. The DNA of 10 positive recombinant clones were PCR amplified using primers of the vector in order to select inserts for sequence analysis.

4- PCR ANALYSIS OF THE COLONIES

Bacterial colonies were lysed at 100°C in PCR a heating block, 10 µl of the lysed bacteria were added to forward and reverse M13 primers in order to amplifiy the inserts. PCR reaction comprises incubation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and elongation at 72°C for 1 min. Colonies showing amplification product of 380pb were selected for further analysis by DNA sequencing.

5- DNA SEQUENCING AND ANALYSIS

The positive clones were sequenced using the chain termination method with ABI PRISM DNA sequencer using the M13 primer. The sequences were analysed by the software and clustal X program[24].

6- CONSTRUCTION OF A BOTIT2 EXPRESSION PLASMID

The pEZZ-18 expression system was used for expression of BotIT2 cDNA as a fusion protein coupled to the two IgG-Binding (Z) domains derived from protein A of *Staphylococcus aureus*. A polymerase chain reaction was conducted with a forward primer, IT2Kpn, and a reverse primer IT2Bam. IT2Kpn contains the following elements: a KpnI restriction site (underlined), and a ATG encoding a methionine residue (boldface) and ten N-terminal codons of BotIT2 coding sequence: (5'-GCGGAGGGTACCC**ATG**GATGGATATATTAAAGGTTATAAAGGTTGC-3').

Primer IT2Bam (5'-GCGGCAGGATCC**TTA**GCAGGTGTTGGTTTCAGGTTTCCATC-3') is an antisense oligonucleotide that contains the following: BamHI restriction site to facilitate the subcloning (underlined), a stop codon (boldface) and the coding for the nine C-terminal residues of BotIT2. The PCR products, obtained with IT2Kpn and IT2Bam, were digested with KpnI and BamHI. The BotIT2 open reading frame obtained this way was ligated into pEZZ-18 vector between the KpnI and BamHI sites, to generate the recombinant expression plasmid. The recombinant insert was sequenced to ensure that the coding sequence of BotIT2 is as expected.

7- CHARACTERIZATION OF THE FUSION PROTEIN

Visualisation of the recombinant toxin was performed by 15% SDS/PAGE acrylamide gels[25]. The proteins were transferred onto PVDF membrane. After incubation in blocking solution (1X PBS, 0.1% Tween-20) for 1h, half of the membrane was immunoblotted for another 1h with anti- protein A monoclonal antibody (dilution:1/50000) at room temperature and the other with anti-BotG50 polyclonal antibody (dilution:1/5000); the membranes were incubated for 1h at room temperature with 1:1000 diluted anti-rabbit IgG conjugated to alkaline phosphate. Diaminobenzidine (DAB) were used as substrate to reveal the Ag/Ac complexes.

RESULTS AND DISCUSSION

1- CLONING AND NUCLEOTIDE SEQUENCE ANALYSIS

To determine the nucleotide sequence of the insect toxin BotIT2, the corresponding cDNA was cloned using rapid amplification of cDNA ends (RACE) PCR. One µg of mRNA isolated from frozen scorpion telsons served as a template for the first-strand cDNA synthesis with the degenerate primer IT2r specific to the C-terminal sequence of BotIT2. 3' RACE PCR amplification of this cDNA was then performed using the oligonucleotide PCR primer based on the N-terminal IT2a and C-terminal IT2r sequence of BotIT2.

The degeneracy was reduced based on information about frequency of codon usages for scorpion toxins. The PCR amplification with the designed degenerate primers IT2a and IT2r resulted in the isolation of PCR fragments of about 180pb (Figure 1) by 2% agarose electrophoresis gel. The DNA fragments were then

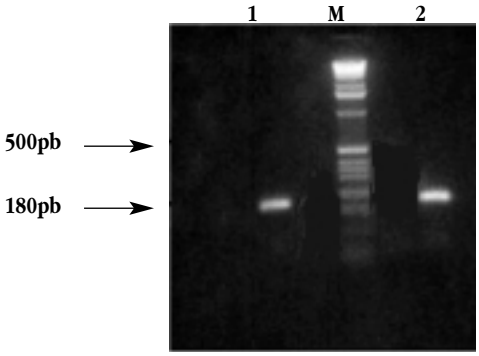

Figure 1: PCR amplification of BotIT2. Lanes (1) and (2) shows the pfu polymerase products of BotIT2 on 2% agarose. (M) 1kb marker.

purified by PCR purification kit (Qiagen) and cloned into the pGEM-T vector (Promega). White clones were screened by PCR with forward and reverse M13 primers (Figure 2), three clones have a positive amplification that correspond to 380pb. The nucleotide sequence was analysed. It displayed an open reading frame (ORF) of 180pb encoding a polypeptide of 60 amino-acids (Figure 3) corresponding to the mature toxin BotIT2.

Compared with the gene sequence of BotIT2 the correct codon in primer IT2a was Asp (GAT), Gly (GGA), Ile (ATT), and in the primer IT2r was Lys (AAG), Arg (AGA), Thr (ACC), Asn (AAC), Cys (TGC).

The cDNA deduced sequence was identical to the amino-acid sequence already established for BotIT2[1]. The nucleotide sequence of BotIT2 and Table I show frequency usage of codons, in the case of Aspartic acid we have dominance for GAT codon with 66.66%, 100% of CTT for the Leucine, 100% of TAT for Tyrosine, 66.66% of ATA for Isoleucine, 85.71% of AAA for Lysine and 60% GAA for Glutamic acid. The usage of these codons and the preference to one codon than to the other could be used as information for the oligonucleotide synthesis of Buthus occitanus tunetanus toxin precursor.

2- EXPRESSION AND HYBRID CHARACTERIZATION

Expression assays were explored for BotIT2. Recombinant BotIT2 was expressed in HB101/ *E. coli* strain, as a fusion protein with two IgG-Binding (ZZ) domains derived from protein A of *Staphylococcus aureus* using the expression vector, pEZZ18[26, 27, 28, 29].

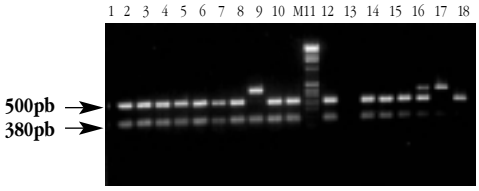

Figure 2: PCR analysis of the clones by forward and reverse M13 primers. The clones 8, 16 and 17 show positive amplification of 380pb.

GAT GGA TAT ATT AAA GGT TAT AAA GGT TGC AAA ATA
D G Y I K G Y K G C K I
ACA TGC GTG ATA AAT GAT GAC TAT TGT GAT ACA GAG
T C V I N D D Y C D T E
TGT AAA GCT GAA GGT GGC ACT TAT GGA TAT TGT TGG
C K A E G G T Y G Y C W
AAA TGG GGA CTT GCC TGT TGG TGC GAA GAT CTT CCA
K W G L A C W C E D L P
GAG GAC AAG AGA TGG AAA CCT GAA ACC AAC ACC TGC
E D K R W K P E T N T C

Figure 3: Nucleotide sequence encoding BotIT2 precursor and the deduced amino-acid sequence.

This is a secretion vector designed for the translocation of Z-domain containing fusion proteins into the growth medium of bacteria. Expression plasmid construction was performed after modification by PCR amplification of the cDNA sequence that encodes the precursor sequence of the toxin. The forward oligonucleotide (IT2Kpn) allowed introduction of a KpnI restriction site (underlined), and an ATG codon of methionine residue. Since the mature sequence of BotIT2 has no methionine codon, it constitutes a unique and useful CNBr cliveage site. The reverse oligonucleotide (IT2 Bam) was designed to introduce a BamHI restriction site and the stop codon TAA, which is efficiently recognized in E. *coli*. Thus, the constructed pEZZ/BotIT2r recombinant plasmid contains an ORF, encoding respectively the protein A signal peptide, two IgG-binding domains (14kDa), eight amino-acid linker peptide and BotIT2 (7KDa) mature sequence. SDS/PAGE (15% acrylamide) analysis revealed that an IgG-affinity-purified protein with an apparent molecular mass of 21kD (Figure 4 A) was present in the culture medium. We estimated the yield of the fusion protein as approximately 12 mg/l of bacterial culture.

Table I: Representative frequency of codon in BotIT2 nucleotide sequence

Number of amino acid	Amino acid residue	Codon	Codon usage
6D	Aspartic Acid	GAT	66.66%
		GAC	33.33%
2L	Leucine	CTT	100%
7G	Glycine	GGA	42.85%
		GGC	14.28%
		GGT	42.85%
2P	Proline	CCA	50%
		CCT	50%
5Y	Tyrosine	TAT	100%
3I	Isoleucine	ATT	33.33%
		ATA	66.66%
7K	Lysine	AAA	85.71%
		AAG	14.28%
5T	Threonine	ACA	40%
		ACT	20%
		ACC	40%
1R	Arginine	AGA	100%
1V	Valine	GTG	100%
2N	Asparagine	AAT	50%
		AAC	50%
5E	Glutamic Acid	GAG	40%
		GAA	60%
4W	Tryptophan	TGG	100%
8C	Cysteine	TGC	50%
		TGT	50%
2A	Alanine	GCT	50%
		GCC	50%

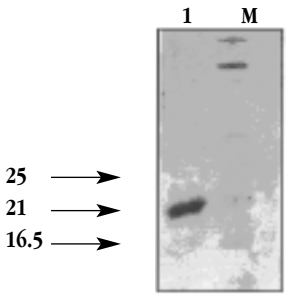

Figure 4: A) SDS PAGE (15%) of periplasmic proteins purified on an IgG-Sepharose column.
Lane 1, purified recombinant hybrid ZZ/BotIT2 from culture medium fraction of Hb101 host transformed with pEZZ/BotIT2; Lane M, molecular weight markers (kDa).

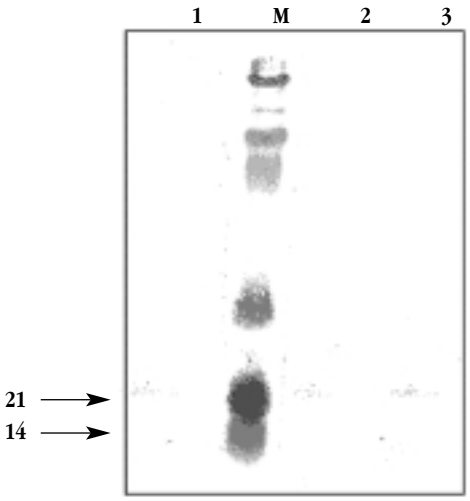

Figure 4: B) Western blot analysis of BotIT2r in Hb101 transformed cells.
Lane(1) with anti-protA, Lanes (2,3) with anti-BotG50; lane M, Rainbow molecular markers.

The specificity of the hybrid pEZZ/BotIT2r is demonstrated by western blot (fig4 B), using a monoclonal anti-protein A (lane 1) and the polyclonal anti-BotG50 (lane 2,3). The IgG purified fusion protein was cleaved by CNBr (data not shown). In the first assay of cleavage we were not able to get purified toxin. Therefore we have to improve conditions of cleavage and purification to get an active toxin. We may also change chemical cleavage (CNBr) by enzymatic cleavage, such as using Factor Xa[29] or enterokinase. Cloning, expression of BotIT2 toxin and the substitution of critical amino acid is essential to understand its action on the activation kinetics on insect sodium channels. Specific mutagenesis should lead us to understand the relationship between toxicity and localisation of particular specific functional sites.

In this work, we isolated a cDNA sequence encoding insect toxin 2 from *Buthus occitanus tunetanus.* Expression of BotIT2 was realized as fusion protein in E. *coli*, purification of cleaved toxin is underway. Other alternatives to toxin purification ways are explored. Our study could also lay foundation for further studies of structure-function relationship by carrying out specific amino-acid substitutions in toxin molecules through recombinant techniques.

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