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Development of a bacterial cloning vector for expression of scorpion toxins for biotechnological studies

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Abstract

Scorpion venoms contain toxic peptides that recognize K^+ channels of excitable and non-excitable cells. These toxins comprise three structurally distinct groups designated α-KTx, β-KTx, and γ-KTx. It is highly desirable to develop systems for the expression of these toxins for further physiological and structural studies. In this work, an expression vector (pTEV3) was constructed by inserting protein D (major capsid of phage lambda) and TEV protease recognition site into plasmid pET21d DNA sequences. Three α-KTx toxins (OsK2, PbTx1, and BmKK3) were cloned into vector pTEV3 and expressed as soluble fusion proteins. The fractions containing the purified fusion proteins (protein D–toxin) were treated with TEV protease to remove protein D. The resulting toxins were analyzed by MALDI-TOF Mass Spectrometry. The results showed that the vector is appropriate for the expression of the target toxins in soluble form and that ion exchange purification of these toxins by flow-through recovery is possible. Analysis by MALDI-TOF Mass Spectrometry of Osk2 demonstrated that this toxin was expressed in its native form, as suggested by the values expected for the presence of two disulfide bridges.

Keywords: Molecular cloning; Toxin; Scorpion

Scorpion venoms, a rich source of several classes of peptides, affect the function of ion channels [1–3]. They disrupt the normal function of excitable tissues such as muscles and nerves, causing respiratory and circulatory damages that can eventually lead to death. Despite the public health problems caused by scorpion stings, these peptides are useful ion channel characterization and neurobiological study tools [1,2]. Several toxins have been extensively studied with respect to their structure, mode of action, and localization of functional sites. The most widely known toxins are those specific for sodium and potassium channels [4–6]. The toxic peptides that recognize K^+ channels of excitable and non-excitable cells [7,8] comprise three structurally distinct groups designated α-KTx, β-KTx, and γ-KTx [8–10]. The α-KTx group includes peptides containing 28–40 residues with three or four disulfide bridges [8]. A systematic nomenclature was proposed for α-KTx based on 12 independent sub-families [8], which were later increased to 20 [11]. These peptides modify potassium cell membrane permeability by recognizing and blocking the proteins responsible for forming the K^+ channels by physically occluding the channel pore [7,12]. Minute amounts of peptide are involved in this process, usually less than 1% of the injected venom.

The purpose of the present work was to develop a system for cloning and expressing scorpion venom toxins in an easily purifiable soluble form for use in studies involving circular dichroism, NMR spectroscopy and biological activity. For this, three α-KTx toxins (OsK2 from *Orthochirus scrobiculosus*, PbTx1 from *Parabuthus*

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tranvaalicus, and BmKK3 from *Buthus martensii*) were cloned in plasmid vector pET21d after modifications by insertion of nucleotide sequences of protein D (major capsid protein of bacteriophage lambda) and the recognition site for protease TEV (catalytic domain of the protein encoded by the tobacco etch virus). Subsequently, the recombinant plasmid was transformed into the *Escherichia coli* BL21 (DE3) strain, and the expressed proteins were purified by affinity and ion exchange and reverse phase chromatography and analyzed by MALDI-TOF mass spectrometry.

Material and methods

Construction of pTEV3 vector

Vector pTEV3 was constructed using plasmid pET21d (Novagen), which was modified by insertion of DNA sequences of protein gpHD (His-tagged fusion protein) and TEV protease recognition site (for further protein separation). Initially, the nucleotide sequence of protein D was inserted between NcoI and BamHI restriction sites of pET21d as performed by Forrer and Jaussi [13]. Subsequently, a hybrid nucleotide containing DNA sequences for TEV recognition site and toxin TsTx-IV (a gift from Dr. Frank Bernard) flanked by BamHI and HindIII restriction sites was cloned in the BamHI and HindIII sites of this plasmid, resulting in plasmid pTEV3. This construction was used as a control to verify its capacity to drive the expression and purification of a known toxin in soluble form. The hybrid nucleotide was sequenced using T7 universal primers to verify all the cloning experiments. After this, the entire sequence (TEV:TsTx-IV) was removed with enzymes BamHI and HindIII and a synthetic 34-pb fragment (corresponding to the nucleotide sequence of the seven amino acid residues ENLYFQG) encoding just the TEV protease recognition site was inserted into the pTEV3 vector. This fragment possesses BglII restriction site at the 5′ end and BamHI and HindIII restriction sites separated by four bases (ATCG) at the 3′ end (Fig. 1). This construction allowed the insertion of the TEV recognition site and the maintenance of BamHI and HindIII restriction sites for cloning toxin nucleotides upstream the TEV recognition site. The single-strand polynucleotides used to construct the TEV recognition site after annealing are exhibited in Fig. 1.

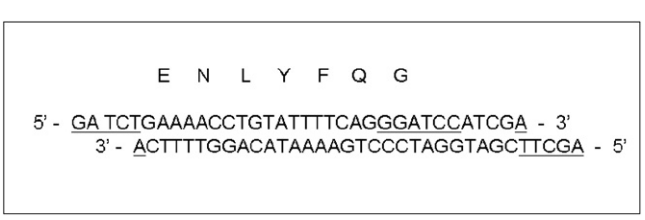

Fig. 1. Nucleotide and amino acid sequence for the TEV recognition site. The BglII and the BamHI restriction sites at 5′ and 3′, respectively, are underlined.

Synthesis of the toxin oligonucleotides and cloning into pTEV3

The synthesis of toxin oligonucleotides was accomplished as described by Prodromou and Pearl [14]. Recombinant OsK2, PbTx1, and BmKK3 toxins are composed of 28, 37, and 31 amino acids, respectively. The peptide sequences of these toxins were converted to nucleotide sequences taking in consideration the codons found most frequently in *E. coli*. In order to prepare the toxin coding sequences, six DNA fragments (approximately 35 bases each) were synthesized for BmKK3 toxins and four DNA fragments (approximately 35 bases each) for OsK2 and PbTx1 toxins. All DNA sequence coding was made through hybridization steps in such a way that each nucleotide pair of the double-strand had eight unpaired bases in one of the strands, which would allow the hybridization with the eight unpaired bases of the next nucleotide pair, as observed by DNA sequences for the construction of toxin OsK2: 5′ ga tcc gcg tgc ggc ccg ggc tgc agc ggc agc tgc cgc cag aaa ggc g 3′–3′ g cgc acg ccg ggc ccg acg tcg ccg tcg acg gcg gtc ttt ccg cta gcg taa 5′ and 5′gat cgc att aaa tgc att aac ggc agc tgc cat tgc tat ccg taa a 3′–3′ ttt acg taa ttg ccg tcg acg gta acg ata ggc att ttc ga 5′. Terminal fragments were flanked by BamHI and HindIII sites at 5′ and 3′ ends, respectively, to facilitate cloning into the pTEV3 vector. A stop codon (TAA) was also included in the 3′ end. The synthetic oligonucleotides were initially phosphorylated by treatment with T4 DNA Polynucleotide Kinase (Amersham Biosciences) in the presence of 5 mM of ATP. Fragment pairs, i.e., 1 and 2, 3 and 4, and 5 and 6, in concentration of 2 μM each were annealed by heating at 94 °C for 5 min, followed by slow cooling to 25 °C. Taq DNA ligase (40 U) and 500 ng of the plasmid vector (pTEV3) were added to the mixture of fragments to allow ligation at 16 °C for 18 h. The vector was then transformed into *E. coli* strain DH5α and the plasmidial DNA content of recombinant cells was analyzed as described by Sambrook et al. [15]. DNA sequence analysis was determined using the PE Biosystem Model 377 DNA sequence with the universal T7 promoter primers.

Protein expression and purification

After transformation into the *E. coli* strain BL21 (DE3) pLysS for plasmid propagation, cells were grown at 25 °C in Luria–Bertani broth [15] containing 50 μg/mL of ampicillin. When cultures reached an OD600 from 0.8 to 0.9, 0.5 mM isopropyl thio-β-D-galactoside (IPTG)[1] was added to the culture broth for induction for 4 h. After induction, cells were harvested, disrupted by sonication, and centrifuged. Analysis of the expressed protein was verified by polyacrylamide gel electrophoresis as described by Sambrook

[1] *Abbreviations used:* IPTG, isopropyl thio-β-D-galactoside; CD, circular dichroism; Mw, molecular weight.