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Heterologous expression of five disulfide-bonded insecticidal spider peptides

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ABSTRACT

The genes of the five disulfide-bonded peptide toxins 1 and 2 (named Oxytoxins or Oxotoxins) from the spider *Oxyopes lineatus* were cloned into the expression vector pQE30 containing a 6His-tag and a Factor Xa proteolytic cleavage region. These two recombinant vectors were transfected into *Escherichia coli* BL21 cells and expressed under induction with isopropyl thiogalactoside (IPTG). The product of each gene was named HisrOxyTx1 or HisrOxyTx2, and the protein expression was *ca* 14 and 6 mg/L of culture medium, respectively. Either recombinant toxin HisrOxyTx1 or HisrOxyTx2 were found exclusively in inclusion bodies, which were solubilized using a chaotropic agent, and then, purified using affinity chromatography and reverse-phase HPLC (RP-HPLC). The HisrOxyTx1 and HisrOxyTx2 products, obtained from the affinity chromatographic step, showed several peptide fractions having the same molecular mass of 9913.1 and 8030.1 Da, respectively, indicating that both HisrOxyTx1 and HisrOxyTx2 were oxidized forming several distinct disulfide bridge arrangements. The isoforms of both HisrOxyTx1 and HisrOxyTx2 after DTT reduction eluted from the column as a single protein component of 9923 and 8040 Da, respectively. *In vitro* folding of either HisrOxyTx1 or HisrOxyTx2 yielded single oxidized components, which were cleaved independently by the proteolytic enzyme Factor Xa to give the recombinant peptides rOxyTx1 and rOxyTx2. The experimental molecular masses of rOxyTx1 and rOxyTx2 were 8059.0 and 6176.4 Da, respectively, which agree with their expected theoretical masses. The recombinant peptides rOxyTx1 and rOxyTx2 showed lower but comparable toxicity to the native toxins when injected into lepidopteran larvae; furthermore, rOxyTx1 was able to inhibit calcium ion currents on dorsal unpaired median (DUM) neurons from *Periplaneta americana*.

1. Introduction

Cysteine-rich spider venom peptides are emerging as valuable leads with potential therapeutic and agricultural applications (Striessnig et al., 1998; Nakasu et al., 2014). However, because of their limited sources, cysteine-rich spider venom peptides are difficult to obtain; so it is imperative to count on either chemical or biological efficient synthetic processes to gain enough material for further research. Yet the synthetic processes to obtain fully active cysteine-rich spider peptides are difficult because they have to be folded correctly. The primary structures of two interesting cysteine-rich insecticidal spider peptides, OxyTx1 and OxyTx2, from the venom of the spider *Oxyopes lineatus* were earlier reported (Villegas et al., 2008). OxyTx1 and OxyTx2 inhibited voltage-sensitive calcium channels (VSCCs) L-, N- and P/Q-type; so their insecticidal mode of action could be trough shutting down insects' VSCCs. Some interesting features of these two OxyTxs is that they do not have an effect on voltage-gated sodium channels, and they are not toxic to mammals (Villegas et al., 2008).

In this regard, some classical examples of animal venom peptides already reported with insecticidal and therapeutic potential that modify VSCCs are: Ptu1 and Ado1 from the assassin bugs

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Peirates turpis and *Agriosphodrus dohrni*, respectively (Corzo et al., 2001a,b), the ω-atracotoxins from the spider *Hadronyche versuta* (Wang et al., 2001), the ω-agatoxins from the spider *Agelenopsis aperta* (Adams et al., 1990), SNX-482 from the spider *Hysterocrates gigas* (Newcomb et al., 1998), GsTxSIA from the spider *Grammostola spatulata* (Lampe et al., 1993), ω-PnTx3-3 from the spider *Phoneutria nigriventer* (Leão et al., 2000) and ω-MVIIA from the cone snail *Conus magus* (Olivera et al., 1994; Miljanich, 2004).

In this work, two five disulfide-bonded peptides from the venom of the oxyopid spider *O. lineatus* that antagonize VSCCs were heterologous expressed in *Escherichia coli* strain BL21, and they were properly folded *in vitro* conditions. Although OxyTx1 and OxyTx2 differ markedly from each other in their number of residues, their secondary structures contain similar structure scaffolds. The recombinant rOxyTx1 and rOxyTx2 were lethal to lepidopteran larvae, and particularly the recombinant rOxysTx1 affected VSCCs from *Periplaneta americana*.

2. Material and methods

2.1. Bacterial strains, enzymes and plasmids

The DH5-α *Escherichia coli* strain was used for DNA cloning and plasmid propagation. The BL21 *E. coli* strain was used for the expression of the recombinant toxins. Plasmids pBluescriptKS (+) (Stratagene, Amsterdam, The Netherlands) and pQE30 (Qiagen, CA, USA) were used for the cloning of the toxins genes, and production of the 6xHis-tagged recombinant toxins, respectively. Restriction enzymes, *Vent* polymerase, Factor Xa protease (FXa) and T4 DNA ligase were purchased from New England Biolabs (New England Biolabs, MA, USA).

2.2. Gene assembly

Based on the information obtained from the direct peptide sequencing of OxyTx1 and OxyTx2 (Villegas et al., 2008), specific oligonucleotides were designed for *de novo* assembly of their genes. The peptide sequences were back-translated and the codons harmonized to reflect *E. coli* codon usage (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=37762). The recognition sequences for the restriction enzymes used for cloning (BamHI and PstI) and the sequence encoding for the Factor Xa protease cleavage site were also included in the design. Six oligonucleotides were synthesized for the OxyTx1 gene: Oxy1_Up1 (54-mer): 5′-GAGGATCCATCGAGGGAAGGGATTGGGAATGTCTGCCGTTACATAGCTCTTGCG-3′, Oxy1_Lw2 (63-mer): 5′-GGACAATGGCAGTGATGGTTTTTACAGCAAACACAGTCATTATCGCAAGAGCTATGTAACGGC-3′, Oxy1_Up3 (51-mer): 5′-AACCATCACTGCCATTGTCCTTATAGTAATGTGAGCAAACTGGAGAAGTGG-3′, Oxy1_Lw4 (59-mer): 5′-ACGTTTCAGCGCATCTGGAATTTTTGCCCATTCCGGAAGCCACTTCTCCAGTTTGCTCA-3′, Oxy1_Up5 (56-mer): 5′-TCCAGATGCGCTGAAACGTTGCTCATGTCAGCGCAACGATAAGGACGGTAAAATCA-3′, Oxy1_Lw6 (59-mer): 5′-TCCTGCAGTCATTAGTTCTTGTATTTATCGCAGGTATTGATTTTACCGTCCTTATCGTT-3’. The overlap of the complementarity regions ranged from 19 to 21 nucleotides, with estimated Tm's of 63–66 °C. For the OxyTx2 gene six oligonucleotides were also employed: Oxy2_Up1 (50-mer): 5′-GAGGATCCATCGAGGGAAGGGCATGGAAATGTTTACCGAAGGATAGCACC-3′, Oxy2_Lw2 (52-mer): 5′-AGCCTTCACAGCAATCACAGTCATCACCGCAGGTGCTATCCTTCGGTAAACA-3′, Oxy2_Up3 (49-mer): 5′-CTGTGATTGCTGTGAAGGCTTACATTGTCACTGCCCTCTGCGTAATATG-3′, Oxy2_Lw4 (57-mer): 5′-CTTTAGACTGGCAGCTACAGCGCAGAATGGCTGGCAGCATATTACGCAGAGGGCAGT-3′, Oxy2_Up5 (46-mer): 5′-GCTGTAGCTGCCAGTCTAAAGACGATCATATTAACACCTGTCCGAA-3′, Oxy2_Lw6 (48-mer): 5′-TCCTGCAGTCATTAGCTTTTCTTATATTTCGGACAGGTGTTAATATGA-3′, with overlaps of 20–21 nucleotides and Tm's ranging from 63 to 64.5 °C (OLIGO Primer Analysis Software, http://www.oligo.net).

The genes were assembled by recursive PCR. First, the oligonucleotides were combined in pairs (Up1+Lw2, Up3+Lw4, Up5+Lw6) to produce three products that were purified from agarose gels with the QIAquick Gel Extraction Kit (Qiagen, CA, USA) and then mixed together in a fourth PCR reaction to assembly the complete genes. The PCR conditions were as here described: a 3 min denaturation step at 94 °C, after which the polymerase was added (hot start), followed by 30 amplification cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, and a final extension step of 5 min at 72 °C. The agarose gel-purified PCR products were blunt-ligated into EcoRV-digested pBluescriptKS(+), and electroporated into electrocompetent DH5-α bacteria. Positive clones were selected based on the blue/white selection scheme, and the integrity of the constructions was verified using DNA sequencing.

2.3. Plasmid construction for expression

The gene constructions made for OxyTx1 and OxyTx2 were subcloned into the pQE30 expression vector through the BamHI and PstI sites. The pQE30 vector introduces a tandem of six histidines to facilitate purification of the product by affinity chromatography. Also, a Factor Xa protease cleavage sequence was conveniently placed between the 6His-tag and the mature toxin to allow the cleavage of the full recombinant toxin. The new pQE30-derived constructs were verified by sequencing from both sides. Competent *E. coli* BL21 cells were transfected with the corresponding plasmids by incubation for 2 min at 42 °C, followed by 5 min in ice, recovered for 30 min at 37 °C in LB medium and plated in LB containing 100 μg/mL of ampicillin. The constructions were named pQE30XaOxyTx1 and pQE30XaOxyTx2, and their expression products are here abbreviated as HisrOxyTx1 and HisrOxyTx2.

2.4. Expression and purification of HisrOxyTxs

E. coli strain BL21 cells expressing the plasmid pQE30XaOxyTx1 or pQE30XaOxyTx2 were grown in Luria Broth (LB) medium. After the absorbance at 600 nm reached 0.5 of absorption units, the cultures were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 6 h at 21 °C. Cells were harvested by centrifugation (4000×*g* for 20 min) using a Beckman centrifuges model J2-21, recovered in washing buffer (20 mM Tris Buffer, pH 8.0) and lysed with a French pressurizing device (900 psi). This material was centrifuged again (4000×*g* for 20 min). The insoluble fraction was resuspended twice with the washing buffer and centrifuged in the same conditions. The recombinant toxin was extracted from the inclusion bodies using the chaotropic agent guanidinium chloride (6 M GndHCl) containing 0.5 M Tris Buffer (pH 8.0) and centrifuged for 40 min in a refrigerated Hettich Universal 32R centrifuge. The supernatant was purified by affinity column. Purification of the HisrOxyTx1 and HisrOxyTx2 by Ni-NTA (Ni-nitrilotriacetic acid) affinity column chromatography was performed according to the instructions of the manufacturer (Qiagen, CA, USA) using denaturing conditions with solution A (6 M GndHCl in a 0.05 M Tris Buffer, pH 8.0) and solution B (6 M GndHCl in 0.05 M Tris Buffer containing 400 mM imidazole, pH 8.0). The imidazole and other salts were eliminated by a second purification step under reverse-phase HPLC (rpHPLC) system using an analytic C_{18} rpHPLC column (Nacalai-Tesque, Japan) and a elution gradient from 20% to 60% solvent B for 40 min. Solvent A was 0.1% trifluoroacetic acid (TFA) in water and solvent B was 0.1% TFA in acetonitrile (Corzo et al., 2007). The HisrOxyTx products were vacuum dried and