Expression of a recombinant Phoneutria toxin active in calcium channels

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A B S T R A C T

PnTx3-4 is a toxin isolated from the venom of the spider Phoneutria nigriventer that blocks N-, P/Q-, and R-type voltage-gated calcium channels and has great potential for clinical applications. In this report we used the SUMO system to express large amounts of recombinant PnTx3-4 peptide, which was found in both soluble and insoluble fractions of bacterial extracts. We purified the recombinant toxin from both fractions and showed that the recombinant peptide showed biological activity similar to the native PnTx3-4. In silico analysis of the primary sequence of PnTx3-4 indicated that the peptide conforms to all the criteria of a knottin scaffold. Additionally, circular dichroism spectrum analysis of the recombinant PnTx3-4 predicted that the toxin structure is composed of approximately 53% turns/unordered, 31% α-helix and 16% β-strand, which is consistent with predicted model of the PnTx3-4 knottin scaffold available at the knottin database (http://knottin.cbs.cnrs.fr). These studies provide the basis for future large scale production and structure-function investigation of PnTx3-4.

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1. Introduction

Peptide toxins obtained from animal venoms are resourceful compounds to investigate ion channels, contributing to our understanding of key channels regulating excitability of neurons and cardiomyocytes. Toxins obtained from the venom of different spiders and sea snails have provided the framework to understand the structure–function relationship of a variety of channels including calcium, potassium, sodium and ligand-gated channels (Doering and Zamponi, 2003; Li and Tomaselli, 2004; Castellino and Prorok, 2000; Lewis et al., 2000; Favreau et al., 1999). Peptide toxins have also been used as potential lead compounds for the development of novel therapeutic drugs (Alonso et al., 2003; Heading, 2002; Jones and Bulaj, 2000; Livett et al., 2004; Lewis, 2009). Importantly, a synthetic neuroactive peptide equivalent to the ω-conotoxin MVIIA, one of the toxins that target voltage-gated calcium channels, has been approved for the treatment of pain (Williams et al., 2008).

Calcium is essential in many physiological mechanisms including hormone and neurotransmitter release, muscle contraction and gene transcription; however, excess calcium influx can generate a cascade of events that cause cytotoxicity and cell death, making calcium a key player in ischemic neuronal death (Lau and Tymianski, 2010;
Arundine and Tymianski, 2003; Sattler and Tymianski, 2000). After an ischemic injury, calcium floods into neurons through different channels including voltage-gated calcium channels, ionotropic glutamate receptors such as N-methyl-D-aspartate (NMDA) and z-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors (Lau and Tymianski, 2010). Therefore, there is an intensive search for calcium channel blockers and glutamate receptors antagonists in the attempt to develop novel neuroprotective drugs (Domin et al., 2010; Lipton, 2007, 2006).

The venom of the Brazilian ‘armed’ spider Phoneutria nigriventer has a number of peptides that are effective blockers of distinct calcium, potassium and sodium channels (de Castro Junior et al., 2008; Vieira et al., 2007, 2005; Cardoso et al., 2003; Carneiro et al., 2003; Vieira et al., 2003; Reis et al., 2000; Penaforte et al., 2000; Reis et al., 1999; Kushmerick et al., 1999; Mesquita et al., 1998; Kalapothakis et al., 1998b, 1998a; Moura et al., 1998; Miranda et al., 1998; Guatimosim et al., 1997; Prado et al., 1996). Three of these toxins, named PnTx3-3, PnTx3-4 and PnTx3-6 are voltage-gated calcium blockers that interfere with the release of glutamate from isolated nerve terminals (Carneiro et al., 2010; Prado et al., 1996; Souza et al., 2007, 2005; Reis et al., 1999). PnTx3-4 irreversibly inhibits P/Q and N-type channels, whereas its action against R-type channels is incomplete and reversible (Dos Santos et al., 2002). PnTx3-3 and PnTx3-6 reversibly and non-specifically inhibit a broad spectrum of high-voltage-activated Ca2+ channels, namely L-, N-, P/Q-, and R-type, with varying potency (Vieira et al., 2005, 2003; Leao et al., 2000). Recent studies have suggested that these peptides can interfere with processes related to ischemia-induced glutamate release and responses to pain (Dalmolin et al., 2011; Agostini et al., 2011; Pinheiro et al., 2009; Souza et al., 2008). These three peptides decrease glutamate release as well as neuronal cell death in retina slices submitted to ischemic injury (Agostini et al., 2011). Additionally, PnTx3-3 and PnTx3-6 have been shown to be effective for the control of neuropathic pain in animal models with no adverse motor effect (Dalmolin et al., 2011; Souza et al., 2008); PnTx3-4 attenuates neuronal death and electrophysiological consequences of oxygen and glucose deprivation in brain slices (Pinheiro et al., 2009); and PnTx3-6 has analgesic effects in rodent models of chronic and acute pain (de Souza et al., 2011; Souza et al., 2008). Therefore, these peptides have the potential to be used in the therapeutic management of pain and/or as neuroprotective drugs.

Purification of toxins from P. nigriventer venom is an expensive, inefficient and time-consuming process. Moreover, the yield for most toxins present in the venom is very low (Cordeiro et al., 1993), making it difficult to completely characterize these peptides. Furthermore, pharmacological use of these peptides will only be feasible if they can be produced in large scale. Generation of recombinant toxins using Escherichia coli is an alternative approach and has been used previously to obtain functional recombinant toxins from the P. nigriventer spider (Souza et al., 2008; Carneiro et al., 2003). In this study, we demonstrate for the first time the functional expression of the toxin PnTx3-4, a valuable scaffold for the development of new neuroprotective drugs.

2. Materials and methods

Oligonucleotides used for PCR reactions were synthesized by Sigma. Restriction endonucleases were purchased from New England Biolabs. The pE-SUMO LIC vector and SUMO protease I were obtained from LifeSensors Inc. (Malvern, USA). ORIGAMI (DE3) competent cells were supplied by Novagen Inc. (Madison, USA). Acetonitrile, Fura-2-AM, glutamate dehydrogenase and Percoll were obtained from Sigma Chemical Co. (MO, USA).

2.1. Plasmid construction

Four oligonucleotides named Tx34A53, Tx34A55, Tx34B53 and Tx34B35 were used as template for the PCR reaction that produced the coding region for the PnTx3-4 toxin (Table 1). Another two oligonucleotides, Tx34SUMOF and Tx34SUMOR (Table 1) were used as primers of the same reaction. BsaI and XbaI restriction sites (underlined) were added at the 5’ and 3’ ends of the DNA, respectively, and a stop codon (TCA) was also added at the 3’ end in order to stop translation after the last amino acid of the toxin. The amplification reaction contained the template oligonucleotides in a 0.1 μM concentration, the amplification primers in a 1 μM concentration, 250 μM of each deoxynucleotide triphosphate and 2 U of the thermostable recombinant Taq polymerase. The reactions were subjected to initial denaturation of 4 min at 95 °C and subsequent 40 cycles that consisted of denaturing the DNA at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min.

The PCR product was purified using the QiAquick TM Gel Extraction kit (Qiagen, USA), digested with BsuI and XbaI and cloned into pE-SUMO LIC Vector. The plasmid obtained (6xHis-SUMO-PnTx3-4) encodes a fusion protein composed of an 18.0 kDa Yeast SUMO protein (Smt3) fused at the N-terminal with a 6xHis tag and at the C-terminal Table 1

<table>
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<tr>
<th>Name</th>
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<th>Size (bp)</th>
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<tr>
<td>Tx3-4SUMOR</td>
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<td>34</td>
</tr>
<tr>
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<td>120</td>
</tr>
<tr>
<td>Tx34A35</td>
<td>5’-TTTATTTACGCTGTTGTTAGTGTGTTTTTCTG CCGTTAAAAAGTGATGTCCAGTTGGTGGTTGATTTGTTGTTATACACCCACCAAAAAACGACACCAAGGACACAAAG GCCTTATACGACGACACATGCAGACATCACATTTTTTACATCACAAAAATACACCAAGGTTGGTAA CGAATTC3’</td>
<td>120</td>
</tr>
<tr>
<td>Tx34B53</td>
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<td>120</td>
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<tr>
<td>Tx34B35</td>
<td>5’-TTTATTTACGCTGTTGTTAGTGTGTTTTTCTG CCGTTAAAAAGTGATGTCCAGTTGGTGGTTGATTTGTTGTTATACACCCACCAAAAAACGACACCAAGGACACAAAG GCCTTATACGACGACACATGCAGACATCACATTTTTTACATCACAAAAATACACCAAGGTTGGTAA CGAATTC3’</td>
<td>120</td>
</tr>
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with the PnTx3-4 toxin (8.0 kDa). The sequence of the recombinant plasmid was confirmed by automatic sequencing using the dyeoxynucleotide chain-termination reaction (Sanger et al., 1977).

2.2. Expression and purification of the fusion protein

*E. coli* ORIGAMI (DE3) cells containing pGro7 chaperone plasmid were transformed with 6xHis-SUMO-PnTx3-4. An isolated colony was inoculated in 10 mL of LB medium supplemented with Ampicillin (100 µg mL⁻¹) and Chloramphenicol (20 µg mL⁻¹) for cultivation at 30 °C overnight. The culture was then diluted 100-fold in 1 L of LB medium (with antibiotics) containing 0.5 mg mL⁻¹ of l-arabinose (for chaperones expression) and cultured to an OD₆₀₀ of 0.5–0.8. The expression of the recombinant fusion protein was induced with 0.6 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), overnight, at 18 °C with shaking. Cells were harvested by centrifugation at 5000 × g for 15 min, suspended in 30 mL of Resuspension buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing protease inhibitor cocktail (CALBIOCHEM), lysed by passing twice through a French Press (16,000–18,000 psi) and cell debris were removed by centrifugation.

The recombinant toxin expressed in the supernatant was purified by affinity chromatography using a Ni-NTA agarose resin (Qiagen). After purification, elutions were pooled and dialyzed against 20 mM Tris–HCl, 150 mM NaCl, pH 7.5 for 24 h to remove imidazole. To purify the recombinant toxin present in inclusion bodies, the pellet obtained after cell lysis was solubilised in a denaturing buffer (6 M Guanidine–HCl, 100 mM NaH₂PO₄, 10 mM Tris, 20 mM Imidazole pH 8.0) and incubated for 1 h at RT. The sample was then centrifuged at 32,000 g for 30 min and the supernatant was purified by affinity chromatography using a Ni-NTA agarose resin. The elutions were dialedyzed first against 1 M Guanidine–HCl, 0.05 M Tris, 0.15 M NaCl, pH 8.0 and later against 0.1 M Gnd-HCl, 0.05 M Tris, 0.15 M NaCl, 1 mM DTT, pH 8.0.

2.3. Cleavage of 6xHis-SUMO-PnTx3-4 fusion protein and purification of the recombinant PnTx3-4 protein

Samples of 6xHis-SUMO-PnTx3-4 were incubated with SUMO protease I (1 U:100 µg) for 1 h at 30 °C and then incubated overnight at 4 °C to allow complete cleavage. DTT was added to the reaction to a final concentration of 2 mM. The recombinant PnTx3-4 was then purified from the 6xHis-SUMO-tag and protease by C8 Reverse Phase-HPLC using a CH3CN discontinuous gradient in 0.1% TFA. The absorbance was detected at 214 nm. For the PnTx3-4 isolated from the supernatant, peaks corresponding to the recombinant toxin were pooled, freeze-dried and stored at −20 °C until needed. For the PnTx3-4 isolated from the pellet, peaks corresponding to the recombinant toxin were pooled and treated for refolding.

2.4. Refolding

The pure PnTx3-4 lyophilized was resuspended in 6 M Gnd-HCl, 50 mM Tris, pH 8.0 and the disulfide bonds were reduced with 10 mM DTT for 4 h at RT. Before the refolding, the DTT was removed from the sample by filtration using VIVASPIN 6 (3 kDa MWCO). The sample was diluted 20 times into the refolding buffer (550 mM Gnd-HCl, 440 mM l-arginine, 55 mM Tris, 21 mM NaCl, 0.88 mM KCl, 1 mM EDTA, 1 mM GSH and 1 mM GSSG, pH 8.2) to a final protein concentration of 0.1–0.2 mg/mL. The recombinant toxin was added in 5 aliquots with a 2 min interval between each one to minimize the precipitation of folding intermediates. The reaction was performed at 4 °C for 24 h. For desalting and to check the refolded recombinant toxin HPLC profile, the sample was submitted to a C18 Reverse Phase Chromatography and the elution samples were lyophilized and kept at −20 °C until needed.

2.5. Protein analysis

All purification steps were followed by SDS-PAGE and Western blotting. Proteins were resolved on 4–20% gradient gels (Lanza) and stained with RAPIDstain Reagent (CALBIOCHEM) or transferred to a PVDF membrane (Millipore). The membrane was incubated overnight at 4 °C with anti-*P. nigriventer* total venom peroxidase antibodies (1:1250) and developed with ECL Plus Western blotting Detection System (Amersham).

2.6. Preparation of synaptosomes

All experiments were carried out in compliance with the Canadian Council of Animal Care (CCAC) guidelines for the care and use of animals. The protocol was approved by the University of Western Ontario Institutional Animal Care and Use Committee (protocol # 2008-127). All efforts were made to minimize the suffering of animals. Cerebral cortices from adult mice (C57BL/6) were isolated and homogenized in 0.32 M sucrose solution containing 1 mM EDTA and 0.25 mM DTT. The homogenate was centrifuged at 1000 g for 10 min at 4 °C and the supernatant was purified by discontinuous Percoll gradient centrifugation as described by (Dunkley et al., 2008) with minor modifications. The synaptosomal fraction was resuspended in Krebs-Ringer-Hepes (KRH) buffer (124 mM NaCl, 4 mM KCl, 1.2 mM MgSO₄, 25 mM HEPES and 10 mM Glucose and adjusted to pH 7.4) to a final concentration of 0.5–1.0 mg/mL for each sample.

2.7. Measurement of [Ca²⁺]ᵢ

Synaptosomes were loaded with 5 µM fura-2AM (stock solution 1 mM in DMSO) for measurements of intrasynaptosomal free calcium concentration ([Ca²⁺]ᵢ). The quantification was performed in a PTI spectrofluorimeter with the fluorescence emission recorded at 510 nm using 340/380 excitation ratio. Synaptosomes were stirred throughout the experiment and maintained at 35 °C. Native and recombinant toxins were added to the synaptosomal suspension 6 min prior to membrane depolarization with 33 mM KCl. Calibration was performed as described by (Prado et al., 1996) using SDS and EGTA for maximum and minimum fluorescence values.
2.8. Measurement of continuous glutamate release

Glutamate release was monitored by measuring the increase of fluorescence caused by NADPH being produced in the presence of NADP+ and glutamate dehydrogenase. At the beginning of each fluorimetric assay, 1 mM of CaCl2, 1 mM of NADP+, and 50 U of glutamate dehydrogenase were added to the suspension. The excitation wavelength was set at 360 nm and the emission wavelength was monitored at 450 nm. Native and recombinant toxins were incubated with the synaptosomes for 30 min prior to each assay. Calcium independent glutamate release was measured by removing CaCl2 and adding 2 mM EGTA to the preparation.

2.9. Statistical analysis

The results were expressed as mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey test (SigmaSTAT) and Kruskal-Wallis ANOVA followed by Dun’s multiple comparison test.

3. Circular dichroism (CD)

To get information about the secondary structure of the toxin PnTx3-4, the CD spectrum of the functional refolded toxin was collected using a spectropolarimeter Jasco-810 (Jasco Corp.) in water. The temperature was kept at 25 °C and the spectrum was measured from 260 nm to 190 nm using a 1 mm path length cell. A minimum of 10 scans were done at a time.

To get an estimation of secondary structures present in the toxin, the data obtained were analyzed using three different algorithms; CDSSTR, CONTIN and SELCON and two reference sets for each (Sreerama and Woody, 2000; Sreerama et al., 1999; Van Stokkum et al., 1990).

4. Results

4.1. PnTx3-4 amino-acid sequence analysis

Fig. 1 shows the amino-acid sequence of the P. nig-riventer PnTx3-4, toxin and its alignment to two related peptides from the spider Agelenopsis aperta that, as PnTx3-4, block N-, P/Q-, and R-type calcium channels. These three peptides share the same number of amino acid residues (76-residues) and are highly conserved in their primary sequence, showing ~70% similarity and ~50% identity. Interestingly, the sequence similarity is observed essentially in the amino-terminal end of the proteins (first 51 amino acid residues) while the carboxy-terminal end does not show either similarity in amino acid sequence or conserved localization of cysteine residues.

4.2. Cloning and expression of the fusion protein

We used the amino acid sequence of PnTx3-4 (Fig. 1), also named ω-Phonotoxin-IIA (Dos Santos et al., 2002; Cassola et al., 1998), to design a synthetic cDNA. The nucleotide sequence was chosen following the E. coli codon usage (Sharp and Li, 1987) to improve expression of the transcript in prokaryotic cells. The designed PnTx3-4 cDNA (Fig. 2A) was generated by PCR using six overlapping oligonucleotides (Table 1; Fig. 2B) and cloned into the pE-SUMO vector (LifeSensors Inc.). The fusion protein generated after expression of the pE-SUMO-PnTx3-4 plasmid included (i) α (His)6 tag followed by (ii) the yeast SUMO protein (Smt3) and, (iii) the PnTx3-4 peptide (Fig. 2C).

Fusion protein expression was induced in the E. coli strain ORIGAMI (DE3) by addition of 0.6 mM IPTG. As shown in Fig. 3A, the 6xHis-Smt3-PnTx3-4 fusion protein was highly expressed. Although a large amount of the recombinant protein was present in the soluble fraction (Fig. 3A, lane 3), considerable amount of the protein was also present in inclusion bodies (Fig. 3A, lane 2). Because soluble proteins have the potential to be in their native conformation, while proteins found in inclusion bodies need to be denatured and refolded to assume their native structure, we chose to purify the recombinant PnTx3-4 from supernatant and pellet separately.

4.3. Purification of PnTx3-4 from the supernatant

The soluble recombinant 6xHis-SUMO-PnTx3-4 was purified from the supernatant by affinity chromatography using a Ni-NTA agarose resin. Purified fraction was dialyzed for 24 h to remove the imidazole. This purification step isolated the 6xHis-SUMO-PnTx3-4 from most of the bacterial proteins (Fig. 3B, lane 5). To specifically separate the PnTx3-4 from its N-terminal 6xHis-SUMO tag we digested the peptide with SUMO Protease I, which recognizes the SUMO structure at the N-terminus of the fusion protein and cleaves the junction (peptide bond linking the last amino acid residue of SUMO to the first amino acid residue of the recombinant peptide). After digestion, PnTx3-4 was then...
purified by reverse phase chromatography in a Waters HPLC system using a discontinuous CH$_3$CN gradient. Two main peaks were observed, one with retention time of about 31 min and the other with retention time of about 41 min (Fig. 3C). Fractions from each peak were pooled and analyzed by SDS-PAGE and Western blot. We determined that the recombinant PnTx3-4 eluted in peak 1, which presented a band of approximately 8 kDa (Fig. 3D, lane 1) that could be recognized by a polyclonal antibody raised against the spider venom (Fig. 3E, lane 2). Approximately 0.6 mg of pure recombinant PnTx3-4 was reproducibly obtained per litter of bacterial culture (Table 2).

4.4. Effect of soluble recombinant PnTx3-4 on glutamate release

To investigate whether the recombinant peptide presented biological activity analogous to native PnTx3-4, we tested it initially in a glutamate release assay (de Castro Junior et al., 2008; Prado et al., 1996). Total glutamate release was measured in mouse cortical synaptosomes depolarized with 33 mM KCl in the presence of 1 mM CaCl$_2$ (Fig. 4A and B). Addition of 16 nM of native PhTx3-4 (purified from the spider venom) decreased glutamate release by 36%. Ca$^{2+}$ removal from the medium, by adding 2.0 mM EGTA before depolarization with KCl, decreased glutamate release by the same magnitude (Fig. 4A and B), corroborating previous findings that native PnTx3-4 effect is mainly restricted to the Ca$^{2+}$-dependent (exocytotic) glutamate release (de Castro Junior et al., 2008). Addition of 16 nM of recombinant PnTx3-4 peptide was able to block glutamate release from cortical synaptosomes as efficiently as the native toxin (Fig. 4A and B).

4.5. Effect of soluble recombinant PnTx3-4 on [Ca$^{2+}$]$_i$

To further test the biological activity of the recombinant PnTx3-4 we investigated its effect on blocking Ca$^{2+}$ channels involved in glutamate release from cortical synaptosomes. To do that, we measured changes in cytosolic Ca$^{2+}$ in fura-2-loaded synaptosomes (Prado et al., 1996).
Synaptosomes depolarized with 33 mM KCl in the presence of 1 mM CaCl$_2$ showed a fast increase in internal calcium concentration (Fig. 4C). Addition of 16 nM of native PnTx3-4 6 min before KCl depolarization inhibited internal Ca$^{2+}$ increase by approximately 30%. Addition of similar concentration of the recombinant PnTx3-4 peptide to the preparation also blocked Ca$^{2+}$ channels, however, the inhibition of internal Ca$^{2+}$ increase observed was smaller (approximately 20% inhibition).

4.6. Purification of PnTx3-4 from inclusion bodies

Because the 6xHis-SUMO-PnTx3-4 fusion protein showed to be highly expressed as inclusion bodies (Fig. 3, lane 2), we chose to improve our purification yield by purifying it from the pellet. To do that, recombinant 6xHis-SUMO-PnTx3-4 present in the pellet was first solubilised in 6 M of Guanidine-HCl (Fig. 5A) and then purified by affinity chromatography using a Ni-NTA agarose resin. After removal of the imidazole by dialysis, the N-terminal tag was cleaved off by digestion with SUMO protease I (Fig. 5B, lane 2). The recombinant toxin was purified by RP-HPLC and two peaks with retention times of about 32 and 41 min respectively were observed (Fig. 5D and E). The peak with 32 min retention time presented one band of 8 kDa that could be recognized by a polyclonal antibody raised against the spider venom (Fig. 5C, lane 1 and 2). This peptide presented no biological activity when tested in the...
4.7. Refolding of PnTx3-4 isolated from inclusion bodies

Our next step was to determine the optimized condition necessary to obtain reliably refolded, biologically active PnTx3-4. To do that, we incubated the recombinant PnTx3-4 in a strong denaturing buffer (6 M Gnd-HCl, 50 mM Tris, 10 mM DTT, pH 8.0) to completely unfold the protein. After 4 h of incubation at RT, DTT was removed by filtration (VIVASPIN 6 column; 3 kDa MWCO). The toxin was then diluted into a refolding buffer to a final concentration of 0.1–0.2 mg/mL. Nine different refolding buffers were tested (Table 3), ranging from strong to weak denaturing conditions. Refolding was allowed to proceed for 24 h at 4 °C, samples were submitted to RP-HPLC and tested. We estimated refolding yields by measuring biological activity using the glutamate release assay as described for experiments in Fig. 4; that is, 16 nM of each refolded peptide was added to mouse cortical synaptosomes prior to depolarization with 33 mM KCl and total glutamate release was measured (Fig. 5F). As our experiments consistently showed that 16 nM of native PnTx3-4 or Ca\(^{2+}\) removal from the medium (by adding 2.0 mM EGTA before depolarization with KCl) decreased glutamate release by the same magnitude (Fig. 4), for these experiments we compared inhibition of glutamate release by each refolded peptide to that of EGTA containing buffer. A refolded sample that presented decrease of glutamate release similar to that of Ca\(^{2+}\) free medium would be considered to have 100% of the peptides properly refolded. As can be seen in Table 3 and Fig. 5F, refolding of PnTx3-4 was suppressed at the lowest and highest denaturant concentrations (buffers 1–4, 8 and 9). Highest PnTx3-4 activity was observed in trial five, which contained 0.5 M Gnd-HCl, 0.4 M l-arginine, 1 mM GSH and 1 mM GSSG. Under these conditions, more than 80% of the solubilised PnTx3-4 was refolded. Approximately 1.5–2 mg of refolded PnTx3-4 peptide was obtained by using trial five conditions (Table 2).

![Fig. 4. Biological effects of the recombinant PnTx3-4. A and B) Effect of the soluble recombinant PnTx3-4 compared to the native toxin in K\(^{+}\)-induced glutamate release in synaptosomes. Synaptosomes were incubated with the recombinant and native toxins for 30 min and then depolarized with 33 mM of KCl. Ca\(^{2+}\)-independent glutamate release was measured in the absence of Ca\(^{2+}\) and in the presence of 2 mM EGTA. The results are shown by mean ± SEM of glutamate release (nmol/mg of protein). C) Inhibition of calcium influx in depolarized synaptosomes. Synaptosomes loaded with fura-2AM were depolarized with 33 mM of KCl after 6 min of incubation with the recombinant and native toxins. The \([\text{Ca}^{2+}]_i\), was calculated by fluorimetric assay and the results are expressed as mean ± SEM of intrasynaptosomal calcium concentration (nM). D and E) The effect of the recombinant PnTx3-4 purified from inclusion bodies in K\(^{+}\)-induced glutamate release in synaptosomes. ANOVA followed by Tukey test were used to determine statistical significance. *P < 0.001, #P < 0.004.](image)
4.8. Circular dichroism spectrum and estimated secondary structures for PnTx3-4

To gather information about the secondary structure of the toxin, we obtained the circular dichroism spectrum of the functional, refolded, recombinant PnTx3-4 (Fig. 6). Analysis of the spectrum using the CDSSTR, CONTIN and SELCON algorithms (Van Stokkum et al., 1990; Sreerama and Woody, 2000; Sreerama et al., 1999) predicted that the toxin structure is composed of approximately 53% turns/unordered, 31% \( \alpha \)-helix and 16% \( \beta \)-strand.

5. Discussion

In this report we provide a method for expression and purification of recombinant PnTx3-4 with native bioactivity. Identifying ideal conditions for heterologous expression of functional PnTx3-4 was rather challenging, even more challenging than finding the conditions to express other P.
nigriventer toxins (Souza et al., 2008; Carneiro et al., 2003; Kushmerick et al., 1999; Torres et al., 2010; Diniz et al., 2006). This difficulty was probably due to the fact that PnTx3-4 requires the formation of a larger number of disulfide bonds than the other peptides present in the P. nigriventer’s venom (Penaforte et al., 2000; Gomez et al., 2002). That is, seven disulfide bonds are necessary to properly fold PnTx3-4 into its native conformation (Figs. 1 and 7). Initial attempts using expression systems that generate His-tag-fusion proteins under the control of the strong T7 promoter (Studier et al., 1990), or the tightly regulated araBAD promoter (pBAD) (Guzman et al., 1995) were not successful. These trials either did not generate fusion proteins in soluble form or the induction of the protein expression was very low (data not shown). Only the SUMO system was suitable to express large amounts of the protein, which was found in both soluble and insoluble form. The SUMO system uses the SUMO protein (Small Ubiquitin-like Modifier) as a fusion partner, improving the solubility of the expressed protein (Marblestone et al., 2006; Malakhov et al., 2004; Butt et al., 2005). In addition, we co-expressed the chaperones GroEL and GroES to improve the protein folding process (Thomas et al., 1997), and carried out the expression in the ORIGAMI (DE3) strain, which has an oxidizing cytoplasm that provides a better environment for the proper folding of the toxin (Stewart et al., 1998). It has been shown that expression of disulfide rich peptides in ORIGAMI (DE3) strain substantially improve the yield of active proteins purified (Prinz et al., 1997).

Only part of the recombinant PnTx3-4 was expressed as a soluble protein. The yield of soluble PnTx3-4 after all the purification steps ranged from 0.5 to 0.8 mg/L, which is in the same range to what has been reported for other animal toxins successfully expressed in E. coli (Johnson et al., 2000; Meng et al., 2011; Che et al., 2009; Souza et al., 2008; Carneiro et al., 2003). More importantly, the soluble recombinant protein showed biological activity very similar to the native PnTx3-4, both in the glutamate release assay as well as in the measurement of intrasynaptosomal free calcium concentration. These results indicate that, similar to the native peptide, soluble recombinant PnTx3-4 is able to block Ca\(^{2+}\) channels involved in glutamate release from cortical synaptosomes.

Because most of the recombinant PnTx3-4 aggregated as inclusion bodies we also searched for conditions to provide efficient refolding of the insoluble recombinant PnTx3-4. Finding the exact conditions to renature proteins is usually time-consuming as refolding conditions for individual proteins vary considerably (Singh and Panda, 2005; Lilie et al., 1998). The basic protocol requires that purified inclusion bodies are first solubilised with a strong denaturant, such as guanidine hydrochloride (GdnHCl), to produce a completely unfolded protein. DTT is also added to allow reduction of disulfide bridges (Fahnert et al., 2004). The solubilised protein is then diluted or dialyzed into a refolding buffer to reduce the denaturant concentration, allowing the protein to refold based on the information contained in its primary sequence. As the denaturant is removed, protein aggregation tends to compete with renaturation therefore, it is crucial to identify the ideal milieu to recover maximal amounts of native protein. Several factors influence renaturation/aggregation during refolding including protein concentration, concentration of strong and weak denaturants, pH, temperature, and the redox environment (Fahnert, 2004; Lilie et al., 1998). Out of 9 different buffer conditions (Table 3) that we tried, only buffer 5, which contained 0.5 M Gdn-HCl, 0.4 M L-arginine, 1 mM GSH and 1 mM GSSG, allowed proper refolding of PnTx3-4. Using buffer 5 we managed to obtain 1.5–2.0 mg/L of PnTx3-4 refolded after purification from inclusion bodies. Importantly, the refolded peptide also showed biological activity very similar to the native peptide. These results indicate that a balanced molar ratio of reduced to

### Table 3

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Gdn-HCl (M)</th>
<th>L-arginine (M)</th>
<th>Redox environment</th>
<th>Percent refolded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2 mM GSH 0.2 mM GSSG</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0.4</td>
<td>2 mM GSH 0.4 mM GSSG</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0.8</td>
<td>1 mM GSH 1 mM GSSG</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>0.55</td>
<td>0</td>
<td>2 mM GSH 0.4 mM GSSG</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>0.55</td>
<td>0.4</td>
<td>1 mM GSH 1 mM GSSG</td>
<td>82.5%</td>
</tr>
<tr>
<td>6</td>
<td>0.55</td>
<td>0.8</td>
<td>2 mM GSH 0.2 mM GSSG</td>
<td>42.5%</td>
</tr>
<tr>
<td>7</td>
<td>1.1</td>
<td>0</td>
<td>1 mM GSH 1 mM GSSG</td>
<td>62.5%</td>
</tr>
<tr>
<td>8</td>
<td>1.1</td>
<td>0.4</td>
<td>2 mM GSH 0.2 mM GSSG</td>
<td>12.5%</td>
</tr>
<tr>
<td>9</td>
<td>1.1</td>
<td>0.8</td>
<td>2 mM GSH 0.4 mM GSSG</td>
<td>17.5%</td>
</tr>
</tbody>
</table>

*Each buffer contains 55 mM Tris, 21 mM NaCl, 0.88 mM KCl, 1.0 mM EDTA, pH 8.2.*
oxidized thiol reagents (glutathione) was essential to provide the appropriate redox potential to allow formation and reshuffling of disulfide bonds (Misawa and Kumagai, 1999; Wetlauer et al., 1987). Proper renaturation of PnTx3-4 also relied on the presence of non-denaturing concentrations of the chaotrope GdnHCl. Low concentrations of GdnHCl or urea have been suggested to contribute to refolding of proteins by slowing down the refolding kinetics and as a consequence, shifting the competition between renaturation and aggregation toward the renaturation reaction (Fahnert et al., 2004; Lilie et al., 1998). Additionally, the presence of l-arginine also contributed to efficient renaturation of PnTx3-4. Although the mechanism by which l-arginine facilitates renaturation is still not completely understood, it has been hypothesized that increased solubilization of folding intermediates might be involved (Lilie et al., 1998). It is important to note that, although biological assays indirectly suggest that recombinant PnTx3-4 and the native PnTx3-4 share similar properties, we cannot rule out the possibility that minor structural differences might exist. Future studies including investigating whether recombinant peptides co-migrate with the native toxin on HPLC and comparative mass spectroscopy analysis will be necessary to clarify this issue.

Analysis of the peptide masses of different spider venoms revealed a bimodal molecular weight distribution, with 60–70% of the peptides showing 30–50 amino-acids, and a secondary grouping (less than 10%) showing peptides 60–80 amino acids long (Escoubas, 2006). Structural data, although limited, come mainly from the more abundant short peptides. These studies indicate that short spider peptides show mainly two different structural motifs characterized by different cysteine arrangements and structural features. The most common motif is the “inhibitor cystine knot” (ICK), also named knottin, with a consensus sequence of C1X3–7–C2X3–8–C3X6–7–C4X1–4–C5X4–13–C6, where C represents cysteine residues and X is any amino acid residue. Disulfide bond pairing observed in all molecules of this type follow the arrangement: C1–C4, C2–C5, C3–C6. Spatial structure of peptides with ICK motif is characterized by the presence of a β-hairpin and a peculiar “knot” (origin of its name) (Escoubas, 2006; Vassilevski et al., 2009). The other less prominent structural scaffold for short spider toxins is the DDH (disulfide-directed beta-hairpin) motif, with a consensus sequence C1X5–19 C2X2 (G/P) X2 C3X6–19 C4, and arrangement of disulfide bonds C1–C3, C2–C5 (Vassilevski et al., 2009; Escoubas, 2006). It has been proposed that the DDH motif came earlier in evolution and the ICK scaffold should be considered to be a molecular evolution of the DDH motif (Shu et al., 2002; Wen et al., 2005).

Very few of the longer polypeptides present in spider venoms have been isolated and sequenced to date. In addition, the three-dimensional structure of the few long spider peptides that have been described in the literature remains undetermined (Vassilevski et al., 2009). PnTx3-4 and the closely related peptide ω-Aga-IIIA belong to this class of peptides (Fig. 1) (Goncaves et al., 2011; de Castro Junior et al., 2008; Reis et al., 2000; Yan and Adams, 2000). They both are 76 amino acids long, show similar placement of the cysteine residues, and have overall sequence identity of 70%. These data suggest that the disulfide bonding patterns of the two molecules are likely to be very similar; however, there has been no NMR study on either PnTx3-4 or ω-Aga-IIIA to define their three-dimensional structure.

Recently Kozlov and Grishin (2005), based on the fact that the majority of spider toxins share similarity in cysteine arrangement and disulfide bridge pattern, developed a new algorithm that reliably predicts the three-dimensional structure of the cysteine knot motif based on primary sequence analysis. Interestingly, these authors showed that PnTx3-4 and ω-Aga-IIIA primary structure conform to all the criteria of a knottin scaffold (Kozlov and Grishin, 2005). An automated modeling procedure is now available for predicting the three-dimensional structure of knottins (Gracy and Chiche, 2010, 2011) and a database of structural models for all known knottin sequences is freely accessible from the web site http://knottin.cbs.dtu.dk. Fig. 7 shows the comparison between the knottin database predicted three-dimensional structures of PnTx3-4 and ω-Aga-IIIA toxins. The two peptides show remarkable structural similarity (Fig. 7C), not only at the N-terminal end, where they show high sequence similarity, but also at the C-terminus, where the peptides do not show amino acid sequence similarity or conserved localization of cysteine residues (Fig. 1). Based on the fact that the different steps of the homology modeling were carefully optimized using a large set of knottins with known structures and the accuracy of predicted models was shown to lie between 1.50 and 1.96 Å (Gracy and Chiche, 2010), it is tempting to propose that the predicted model for PnTx3-4 is a close representation of the actual structure of the toxin. In fact, our CD spectrum analysis of the refolded toxin indicated that PnTx3-4 contains predominantly random coil formation, which corroborates the predicted model proposed. The functional expression of recombinant PnTx3-4 and the structural analysis reported here provide the basis for future large scale production and structure-function investigation of this peptide.

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Ethical statement

All experiments were carried out in compliance with the Canadian Council of Animal Care (CCAC) guidelines for the care and use of animals. The protocol was approved by the
University of Western Ontario Institutional Animal Care and Use Committee (protocol # 2008-127). All efforts were made to minimize the suffering of animals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

References


