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Expression and isotopic labelling of the potassium channel blocker ShK toxin as a thioredoxin fusion protein in bacteria

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Abstract

The polypeptide toxin ShK is a potent blocker of Kv1.3 potassium channels, which play a crucial role in the activation of human effector memory T-cells (T_{EM}). Selective blockers constitute valuable therapeutic leads for the treatment of autoimmune diseases mediated by T_{EM} cells, such as multiple sclerosis, rheumatoid arthritis, and type-1 diabetes. We have established a recombinant peptide expression system in order to generate isotopically-labelled ShK and various ShK analogues for in-depth biophysical and pharmacological studies. ShK was expressed as a thioredoxin fusion protein in *Escherichia coli* BL21 (DE3) cells and purified initially by Ni²⁺ iminodiacetic acid affinity chromatography. The fusion protein was cleaved with enterokinase and purified to homogeneity by reverse-phase HPLC. NMR spectra of ¹⁵N-labelled ShK were similar to those reported previously for the unlabelled synthetic peptide, confirming that recombinant ShK was correctly folded. Recombinant ShK blocked Kv1.3 channels with a *K_d* of 25 pM and inhibited the proliferation of human and rat T lymphocytes with a preference for T_{EM} cells, with similar potency to synthetic ShK in all assays. This expression system also enables the efficient production of ¹⁵N-labelled ShK for NMR studies of peptide dynamics and of the interaction of ShK with Kv1.3 channels.

Keywords

ShK; potassium channels; protein expression; isotopic labelling; NMR

1. Introduction

Sea anemones produce many polypeptide toxins and proteins that are potent ion channel blockers and cytolytic. The first potassium channel blocker to be isolated and characterized

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Conflict of interest

Kineta Inc. has licensed a patent on ShK-186 from the University of California and is developing this peptide as a therapeutic for autoimmune disease. The authors MWP and CB are consultants to Kineta Inc.

from a sea anemone was ShK toxin, from *Stichodactyla helianthus* (Castaneda et al., 1995; Pennington et al., 1995), which is a 35-residue peptide containing six half-cystines that form three disulfide bonds (Pohl et al., 1995) (Fig 1A). Its solution structure, determined by NMR spectroscopy (Tudor et al., 1996; Tudor et al., 1998), consists of two short α -helices encompassing residues 14–19 and 21–24, and an N-terminus with an extended conformation up to residue 8, followed by a pair of interlocking turns that resembles a 3_{10} -helix (Fig 1B).

The surface of ShK involved in binding to voltage-activated (Kv) potassium channels has been mapped using alanine scanning and selected synthetic analogues (Pennington et al., 1996a; Pennington et al., 1996b). Alanine scanning mutagenesis identified the conserved dyad Lys 22 and Tyr23 as key functional residues (Fig 1C). Other residues contributing to Kv1.3 binding include Arg11, His19, Ser20 and Arg24 (Pennington et al., 1996a; Rauer et al., 1999). These essential residues were found to be clustered on a surface of the peptide that binds to a shallow vestibule at the outer entrance to the ion conduction pathway and occludes the entrance to the pore (Pennington et al., 1996a; Rauer et al., 1999). To examine this interaction in more detail, the solution structure of ShK (Kalman et al., 1998; Tudor et al., 1996) was docked to a homology model of the Kv1.3 channel based on the crystal structure of the bacterial potassium channel KcsA (Doyle et al., 1998; Rauer et al., 2000), using restrained molecular dynamics simulations guided by data from complementary mutational analyses (Lanigan et al., 2002; Rauer et al., 2000). The model reveals that Lys22 of ShK projects into the ion conduction pathway while Arg11 is in close proximity to His404 in one of the Kv1.3 subunits.

All human T lymphocytes express two types of K^+ channels, the voltage-gated Kv1.3 and the Ca^{2+} -activated KCa3.1 channels, which play crucial roles in human T-cell activation (Leonard et al., 1992; Price et al., 1989). The expression levels of these two K^+ channels are dependent upon the state of T-cell activation and differentiation (Wulff et al., 2003a). Naïve $CD4^+$ or $CD8^+$ T cells initially differentiate into long-lived central memory (T_{CM}) T cells, which then differentiate into terminally-differentiated effector memory (T_{EM}) cells upon repeated stimulation. Kv1.3 channels are significantly up-regulated in activated T_{EM} cells, leading to a heightened sensitivity to Kv1.3 channel blockers (Beeton et al., 2006; Wulff et al., 2003b). Activation of naïve and central-memory (T_{CM}) cells, by contrast, results in up-regulation of KCa3.1 channel expression and decreased sensitivity to Kv1.3 channel blockade (Wulff et al., 2003a). The differential expression of Kv1.3 and KCa3.1 K^+ channels in activated T_{EM} and T_{CM} cells implies that it may be possible to selectively suppress T_{EM} cells using a Kv1.3-specific inhibitor without causing generalized immunosuppression. Kv1.3 blockers therefore constitute valuable new therapeutic leads for the treatment of autoimmune diseases mediated by T_{EM} cells, such as multiple sclerosis (MS) and rheumatoid arthritis (Beeton et al., 2011; Beeton et al., 2006; Chi et al., 2012; Wulff et al., 2003b).

Patch-clamp experiments on cloned potassium channels expressed in mammalian cells revealed that ShK blocked not only Kv1.3 (K_d 11 pM) but also Kv1.1 (K_d 16 pM), Kv1.7 (Kalman et al., 1998) and Kv3.2 channels (Beeton et al., 2005; Yan et al., 2005) in the nanomolar range. This lack of specificity constitutes a potential drawback for the use of ShK as a therapeutic agent. For example, lack of selectivity for Kv1.3 over the neuronal Kv1.1 channels could prove detrimental if ShK were to enter the brain through a compromised blood-brain barrier and induce neurotoxicity. It was therefore essential to develop ShK analogues that are selective for Kv1.3 over Kv1.1 and other K^+ channels. Towards this goal, more selective analogues have been made by the incorporation of non-natural amino acids or adducts; these include ShK-Dap22, in which the critical Lys22 was replaced by the shorter, positively charged, non-natural residue 1,3-diaminopropionic acid (Dap) (Kalman et al., 1998); ShK-F6CA, a fluorescein-labelled analogue of ShK (Beeton et al., 2003); and

analogues with either phospho-Tyr (ShK-186) or phosphono-Phe (ShK-192) attached via a hydrophilic linker (i.e aminoethoxyethoxyacetyl) to Arg1 (Beeton et al., 2005; Pennington et al., 2009). However, these analogues have several potential limitations, for example, ShK-186 and ShK-192 contain non-protein adducts while the phosphorylated residue of ShK-186 is susceptible to hydrolysis. As a result, there is still scope for the development of new analogues with enhanced stability and increased specificity for Kv1.3.

In this study we report the cloning, expression and purification of ShK in *Escherichia coli*. The majority of the expressed protein formed insoluble inclusion bodies but the protein was successfully refolded and the final ShK yields obtained from rich and minimal media were 3 mg/L and 2.5 mg/L, respectively. NMR spectroscopic analyses of ¹⁵N-labelled ShK indicated that the protein was correctly folded while electrophysiological studies showed that recombinant ShK blocked Kv1.3 channels with a similar efficiency to that of the synthetic peptide.

2. Material and Methods

2.1 Construction of pET32a/ShK expression plasmid

DNA encoding the amino acid sequence of ShK and an N-terminal enterokinase cleavage site was synthesized by PCR using two overlapping primers and was cloned into a pET-32a expression vector (Novagen, USA). For efficient protein expression in bacterial cells, the DNA template was designed based on optimized codons for expression in *E. coli*. The forward primer (5'-
CCAAGAGAATTCGATGATGATGATAAACGCAGCTGCATTGATACCATTCCGAAA
AGCCGCTGCACCGCGTTTCAGTGCAAACAT) contained an *EcoRI* restriction endonuclease cleavage site (underlined) and enterokinase recognition site (double underlined). The reverse primer (5'-AAGATCAAGCTTCTAGCAGGTGCCGAGGTTTTGCGGCAAAAGCTCAGGCGA
TATTTTCATGCTATGTTTGCAGTCAAACGCGGT) contained a *HindIII* restriction endonuclease cleavage site (underlined). The PCR reaction mixture contained 50 pmol of each primer and 2.5 U of Taq DNA polymerase in a final volume of 50 µL. PCR amplification was performed by heating at 94 °C for 5 min followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension at 72 °C for 5 min. The amplified PCR product was digested with the restriction endonucleases *EcoRI* and *HindIII* and ligated into the thioredoxin-fusion tag-containing pET-32a vector. The sequence of the Trx-ShK fusion protein was confirmed by bi-directional nucleotide sequencing using the T7 promoter and terminator primers.

2.2 Expression of Trx-ShK fusion protein

E. coli BL21(DE3) cells transformed with the pET-32a-ShK expression vector were grown overnight at 37 °C in Luria-Bertani (LB) medium containing 100 µg/mL ampicillin. 1 L LB broth was inoculated with 1% of overnight culture and incubated at 37 °C with agitation at 200 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.5. The culture was incubated at 18 °C for 1 h before the culture was induced with isopropyl-β-D-thiogalactoside (IPTG, Astral Scientific) at a final concentration of 1 mM. The culture was further grown overnight at 18 °C and the cells were pelleted by centrifugation at 6000 g for 10 min at 4 °C and then stored at – 80 °C until further processing. Expression was analyzed by SDS polyacrylamide gel electrophoresis (12 % Bis-Tris SDS-PAGE gel, Bio-Rad) and the identity of the Trx-ShK fusion protein was confirmed by in-gel digestion with trypsin followed by LC/MS/MS analysis (Joint Proteomics Laboratory, Parkville, Australia).

2.3 Refolding of Trx-ShK fusion protein

The cell pellet was thawed and lysed at room temperature for 30 min in a solution of 5 mL bugbuster master mix (Novagen) per gram of wet cell pellet containing 1 × protease inhibitor cocktail (lacking EDTA) (Roche, Germany). The lysed cells were centrifuged at 20,000 g for 20 min at 4 °C to pellet the inclusion bodies and the pellet was first washed with ice-cold buffer containing 2 M urea, 20 mM Tris, pH 8.0, 0.5 M NaCl, 2 % Triton X-100 and then briefly sonicated before spinning at 20,000 g for 10 min at 4 °C. The cell pellet was washed repeatedly with the urea-containing buffer solution and then with buffer alone. Subsequently, the inclusion bodies were resuspended in solubilization buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 20 mM imidazole, 6 M guanidine hydrochloride, 1 mM DTT) and stirred for 60 min at room temperature. The sample was centrifuged for 20 min at 20,000 g at 4 °C and the supernatant was filtered through a 0.22 µm filter before being loaded onto a metal-chelating affinity column (5 mL Ni Sepharose, GE healthcare, USA) with a flow-rate of 1 mL/min. The column was washed with 2 column volumes of solubilization buffer followed by 10 column volumes of wash buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 20 mM imidazole) containing 1 mM DTT, and 6 M urea. Immobilized protein was refolded on-column with a linear gradient of 6 to 0 M urea and 1 to 0 mM DTT at a flow-rate of 1 mL/min over 50 min at room temperature. The immobilized refolded protein was eluted with 20 column volumes of elution buffer (20 mM Tris, pH 8.0, 0.5 M NaCl, 0.5 M imidazole).

2.4 Purification of recombinant ShK

The Trx-ShK fusion protein was dialyzed extensively against enterokinase cleavage buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 2 mM CaCl₂) at 4 °C for 24 h and then incubated with enterokinase (0.06 % w/w) at room temperature for 48 h. The cleaved protein was loaded onto a C18 reverse-phase high-performance liquid chromatography (RP-HPLC) column (Phenomenex, 100 × 10mm) equilibrated in buffer A (99.9 % water and 0.01 % TFA). Proteins were eluted with an acetonitrile gradient in 0.01 % TFA (0–26 % over 30 min followed by 26–64 % over 5 min). Eluted fractions containing ShK were lyophilized for storage.

2.5 Expression of ¹⁵N-labelled ShK

¹⁵N-labelled Trx-ShK fusion protein was expressed in *E. coli* BL21(DE3) using M9 minimal medium supplemented with 100 µg/mL ampicillin. The M9 medium contained 1 g/L ¹⁵NH₄Cl (Cambridge Isotope Labs) as the sole nitrogen source and 4 g/L glucose as the carbon source. Cultures were grown at 37 °C until mid-log phase (OD₆₀₀ = 0.5) was reached, cooled at 18 °C for 1 h and then induced by adding IPTG to a final concentration of 1 mM. The cultures were incubated overnight with agitation at 18 °C. The cells were pelleted by centrifugation at 6000 g for 10 min at 4 °C, lysed with bugbuster master mix containing protease inhibitors, and protein in the pellet (inclusion bodies) was purified as outlined in sections 2.3 and 2.4 above.

2.6 Determination of recombinant protein concentration, purity and mass

Protein and peptide concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce) with bovine serum albumin as a standard. The BCA assay was calibrated against peptide content by amino acid analysis on several ShK samples (Florey Neuroscience Institutes, Australia). Protein purity was determined by SDS-PAGE on 12 % gels using Coomassie blue staining and analytical RP-HPLC.

Protein mass was determined by electrospray ionisation time-of-flight (ESI-TOF) mass spectrometry using a Waters LCT TOF LC/MS Mass Spectrometer coupled to a 2795 Alliance Separations module and Masslynx software version 4.1 (Waters, Australia). Mass

spectra were created by averaging the scans across each total ion current peak and subtracting background. The mass spectrometer conditions were as follows: ionisation mode electrospray ionisation, desolvation gas flow 550 L/h, desolvation temperature 250 °C, source temperature 110 °C, capillary voltage 2400 V, sample cone voltage 60 V, scan range acquired 100–1500 m/z, scan time: 1 sec, internal reference ions positive ion mode = m/z = 556.2771.

2.7 NMR spectroscopy

NMR spectra were recorded on samples of 0.2 mM unlabelled or 0.6 mM ^{15}N -labelled ShK in 90 % H_2O /10 % $^2\text{H}_2\text{O}$ at pH 4.9. The 1D ^1H NMR spectrum was acquired on a Bruker DRX 600 MHz spectrometer while the 2D $^1\text{H}/^{15}\text{N}$ HSQC and SOFAST-HMQC spectra were acquired on a Varian INOVA 600 MHz spectrometer. Both spectrometers were equipped with cryogenic probes. The water resonance was suppressed using the WATERGATE pulse sequence (Piotto et al., 1992). All spectra were collected at 20 °C and referenced to the water resonance. Spectra were acquired over 16384 (1D) or 4096 (2D) data points, with a ^1H spectral width of 14 ppm. The 1D spectrum was acquired with 1024 scans. The HSQC was acquired with 32 scans per increment using 2048 points in the proton dimension and 192 increments in the ^{15}N dimension. The SOFAST-HMQC spectrum (Schanda and Brutscher, 2005) was acquired with 32 scans per increment using 2048 points in the proton dimension and 40 increments in the ^{15}N dimension. The ^{15}N dimension of the SOFAST-HMQC spectrum was extended to 120 points with linear prediction. The ^{15}N dimension of both spectra was apodized with a shifted sine-bell apodization function and zero filled to 1024 complex points prior to Fourier transformation. The spectral widths were 14.0 and 40 ppm in the ^1H and ^{15}N dimensions, respectively. The 3D ^{15}N -edited NOESY-HSQC spectrum was acquired with a mixing time of 120 ms, 16 scans per increment, 2048 complex points in the acquisition dimension, and 96 and 80 increments in the indirect ^1H and ^{15}N dimensions, respectively.

2.8 Animals, cells and cell lines

L929 mouse fibroblast cells stably expressing mKv1.1 and mKv1.3 channels (Grissmer et al., 1994) were gifts from Dr. K. George Chandy (University of California, Irvine). They were maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10 IU/mL penicillin, 0.1 µg/mL streptomycin, 2 mM L-glutamine, 10 % heat-inactivated fetal bovine serum, and 0.5 mg/mL G418 (EMD Chemicals, Gibbstown, NJ).

Female Lewis rats (8–10 weeks old) were purchased from Harlan-Sprague Dawley (Indianapolis, IN, USA) and housed under pathogen-free conditions with food and water *ad libitum*. Animals were euthanised under a Baylor College of Medicine Animal Use and Care Committee approved protocol for blood, spleen, and thymus collection, as described (Beeton and Chandy, 2007).

Freshly prepared buffy coats were purchased from the Gulf Coast Regional Blood Center (Houston, TX) under a protocol approved by the Baylor College of Medicine Institutional Review Board. Mononuclear cells were isolated from the buffy coats using Histopaque-1077 gradients (Sigma, St Louis, MI) and used immediately.

Ovalbumin-specific T_{EM} cells expressing high levels of Kv1.3 channels upon activation were a gift from Dr. Alexander Flügel (University of Göttingen, Germany) and were maintained in culture as described previously (Kawakami et al., 2005; Matheu et al., 2008).

2.9 Electrophysiological analysis

Experiments were conducted at room temperature in the whole-cell configuration of the patch-clamp technique. The patch pipettes had a resistance of 2–4 M Ω when filled with a solution containing (in mM): 145 KF, 10 HEPES, 10 EGTA, and 2 MgCl₂, pH 7.2, 290 mOsm. The bath solution contained (in mM): 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.2, 300 mOsm. Kv currents were elicited every 30 s by 200-ms depolarizing pulses from a holding potential of –80 mV to 40 mV. K_d values and Hill coefficients were determined by fitting the Hill equation to the reduction of peak current measured at 40 mV.

2.10 T lymphocyte proliferation assays

Proliferation assays were conducted as described previously (Beeton et al., 2001a; Beeton et al., 2005; Matheu et al., 2008). Briefly, mononuclear cells (10⁵/well) or rat ovalbumin-specific T_{EM} cells (5×10⁴/well) were plated into 96-well plates and pre-incubated with the peptide blockers for 45 min at 37 °C in RPMI medium (Invitrogen) supplemented with 10 IU/mL penicillin, 0.1 μ g/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 1% RPMI vitamins, 50 μ M β -mercaptoethanol, and 1% heat-inactivated fetal bovine serum (human cells) or 1% Lewis rat serum (rat cells). Human T lymphocyte activation and proliferation was induced by the addition of 60 ng/mL anti-CD3 antibodies (Clone OKT3, eBioscience, San Diego, CA). Rat splenocytes were stimulated with 1 μ g/mL concanavalin A (Sigma). Rat T_{EM} cells were stimulated with 10 μ g/mL ovalbumin in the presence of irradiated thymocytes as antigen-presenting cells. Cells were cultured for 72 h at 37 °C, 5% CO₂, and [³H] thymidine was added during the last 16–18 h of culture. Cells were lysed by freezing at –20 °C. After thawing, DNA was harvested onto fibreglass filters using a cell harvester (Inotech Biosystems International, Rockville, MD). [³H] thymidine incorporation into the DNA of proliferating cells was measured using a β -scintillation counter (Beckman Coulter, Brea, CA).

3. Results

3.1 Expression of Trx-ShK

The Trx-ShK fusion protein (Fig 2A) was highly expressed upon induction with 1 mM IPTG at 18 °C overnight. SDS-PAGE analysis showed the appearance of an intense band at about 25 kDa (Fig. 2B, lane 2). Protein was expressed as inclusion bodies (Fig 2C, lane 2), with yields of 2 g per L from both LB and M9 media, while only a small portion of the Trx-ShK fusion protein was soluble (18 mg/L of LB and 10 mg/L of M9 media). The identity of the Trx-ShK fusion protein was confirmed by in-gel trypsin digestion followed by LC/MS/MS peptide sequence analysis.

3.2 Refolding and purification of ShK

Inclusion bodies containing the His-tagged Trx-ShK fusion protein were solubilized in denaturant and loaded onto an NTA column, then the bound protein was refolded by gradual removal of denaturant. The partially purified Trx-ShK fusion protein was eluted from the Ni-chelating column (Fig 2D) and then cleaved with enterokinase. The cleaved fusion protein (Fig 2D, lane 2) was purified to homogeneity by RP-HPLC (Fig 3A). Analytical RP-HPLC and SDS-PAGE showed that purified ShK was essentially homogenous (Fig 3B).

High-resolution ESI-TOF analysis (Fig 3C) of recombinant ShK toxin produced an average mass of 4055 $\pm$ 0.01 Da. This value was identical to the theoretical mass of 4055 Da for ShK toxin with all six cysteines engaged in the three native disulfide bonds (Pohl et al., 1995). For the fully ¹⁵N-labelled ShK, ESI-TOF analysis yielded a mass of 4109 $\pm$ 0.00 Da which was also identical to the theoretical mass of 4109 Da. The yields, determined by amino acid

analysis, were about 3 mg/L of unlabelled peptide from LB and 2.5 mg/L of ^{15}N -labelled peptide from M9 media.

3.3 NMR spectroscopy

NMR spectroscopy was used to determine whether recombinant ShK was correctly folded in solution. The 1D ^1H (Fig. 4) and 2D $^1\text{H}/^{15}\text{N}$ HSQC (Fig. 5A) spectra exhibited well dispersed peaks with narrow linewidths, as expected for a small folded protein. Comparison with the published NMR spectral data for synthetic ShK (Fig. 4) (Tudor et al., 1998) demonstrated that the recombinant protein adopted a similar fold to synthetic ShK in solution. Recombinant ShK displayed sharp peaks across the spectrum and a peak at 10.87 ppm corresponding to Thr31, as previously reported for the synthetic peptide (Fig. 4) (Tudor et al., 1998).

The Met21 resonance of ^{15}N -labelled recombinant ShK in the HSQC spectrum (Fig 5A) was significantly weaker than those from most of the residues. Other residues such as Arg1 and Pro8 were not expected to show cross peaks in this ^{15}N - ^1H HSQC spectrum, while the Ser2 and Cys3 resonances were significantly broadened as a consequence of intermediate exchange with the solvent water resonance (Tudor et al., 1998). Assignments of NH cross peaks in the HSQC spectrum were confirmed by recording a 3D NOESY-HSQC spectrum, representative strips from which are shown in Fig S1.

Recently, we showed that SOFAST-HMQC spectra have the potential to recover cross peaks from amides in intermediate exchange with water (Yao et al., 2011). As shown in Fig 5B, a SOFAST-HMQC spectrum gave well-defined, intense cross peaks from Cys3 and Met 21, although the peak from Ser2 was still absent. This result further confirms the correct folding of recombinant ShK. More generally, the SOFAST-HMQC method should be useful for many peptide toxins containing solvent-exposed amides whose cross peaks may be weakened or eliminated by exchange with the water.

3.4 K^+ channel blocking activity

Native and synthetic ShK block Kv1.1 and Kv1.3 channels with similar potencies in whole-cell patch-clamp assays (Beeton et al., 2003; Kalman et al., 1998). To determine whether recombinant ShK also blocked Kv1.1 and Kv1.3 channels with a similar potency to the native and synthetic proteins we measured the ability of recombinant and synthetic ShK to block mouse Kv1.3 (mKv1.3) and mouse Kv1.1 (mKv1.1) channels stably expressed in L929 cells (Fig 6). As expected, synthetic ShK blocked both channels with equivalent potencies ($K_d = 10 \pm 2$ pM on mKv1.3 and 21 ± 3 pM on mKv1.1) and a Hill coefficient of 1. Recombinant ShK exhibited similar affinity for both channels, with K_d values of 25 ± 10 pM for mKv1.3 and 15 ± 5 pM for Kv1.1. The differences between the potencies of recombinant and synthetic ShK against Kv1.3 and Kv1.1 are not statistically significant.

The activity of recombinant ShK on the proliferation of freshly-isolated human peripheral blood T lymphocytes was compared with that of the well-characterized Kv1.3 channel blocker ShK-186 (Beeton et al., 2006; Matheu et al., 2008; Pennington et al., 2009). Recombinant ShK inhibited the proliferation of human T lymphocytes with an IC_{50} of 2.2 ± 0.4 nM, a value very similar to that reported previously for ShK-186 (IC_{50} 4.1 ± 0.4 nM) (Beeton et al., 2006; Kalman et al., 1998). We also tested its ability to inhibit rat T_{EM} and naïve/ T_{CM} lymphocytes; recombinant ShK preferentially inhibited T_{EM} cells proliferation (IC_{50} 738 ± 89 pM) with little effect on the proliferation of naïve/ T_{CM} cells, as was previously found for synthetic ShK (Beeton et al., 2001b).

4. Discussion

We have developed an efficient bacterial expression system for the sea anemone peptide ShK toxin. Following NTA chromatography, cleavage by enterokinase and RP-HPLC, we obtained pure peptide of the correct mass. Enterokinase has the advantage of leaving no extra residues at the N-terminus of the cleaved recombinant ShK peptide, thus allowing for direct comparison with the native ShK peptide. Our patch-clamp and T-lymphocyte proliferation data show that recombinant ShK has essentially identical activities to those of synthetic ShK (Beeton et al., 2001b; Kalman et al., 1998).

The use of a thioredoxin tag and oxidative refolding proved to be a successful strategy for the production of biologically active recombinant ShK. It has been well documented that various solubility tags can improve the expression of disulfide-bond enriched polypeptide toxins. For example, the sea anemone toxin anthopleurin-B (ApB) was successfully expressed in *E. coli* as a fusion protein with the gene 9 product of bacteriophage T7 (Gallagher and Blumenthal, 1992). Once purified, the fusion protein was refolded using a coupled glutathione redox system and then cleaved with staphylococcal protease to release ApB with additional Gly and Arg residues at the N-terminus. The total yield of ApB was about 1 mg/L of culture (Gallagher and Blumenthal, 1992). Similar methods were reported for the expression of a scorpion neurotoxin (Turkov et al., 1997) and the sea anemone toxin ATX II (Moran et al., 2006), with yields of 0.5 mg/L and 1.5 mg/L, respectively. In contrast, the short sea anemone toxin ATX III (renamed Av3), with an N-terminal extension of Gly-Ser-Ser-Met-Ala and fused with thioredoxin, was soluble when expressed in *E. coli* (Moran et al., 2007). The use of an S-tag has been reported to increase the yield of the sea anemone toxin BgK to about 1.8–2.8 mg/L of culture (Braud et al., 2004). Overall, solubility tags and oxidative refolding have been used successfully for the production of various peptide toxins (Quintero-Hernández et al., 2011), some of which can be difficult to synthesize because of the presence of multiple disulfide bonds and the need for efficient oxidative refolding.

The 1D ^1H NMR spectrum of recombinant ShK was essentially identical to that of synthetic ShK, indicating that the recombinant protein was correctly folded (Pennington et al., 1999). Previous NMR studies of synthetic ShK showed that all hydrogen-bonded backbone amides had slow or intermediate exchange rates except for Lys18, Ser20 and Met21, which were rapidly exchanging (Tudor et al., 1998). These residues are located within a loop joining the two α helices of ShK (Fig 1) and it was suggested that this region may be more flexible than the surrounding structure. This may partly explain why Met21 exhibits a weaker than expected peak intensity. ^{15}N relaxation experiments will be carried out in future studies to probe the backbone dynamics of ShK over timescales from ps to μs (Kuang et al., 2006; Yao et al., 2004). The backbone amides of Ser2 and Cys3 were affected by exchange with water at pH 4.9 and 293 K (Tudor et al., 1998). The Cys3 resonance was found to be truncated and the Ser2 resonance was too broad to be detected. This observation was in agreement with our findings, where these two peaks were not observed in the HSQC spectrum of recombinant ^{15}N -labelled ShK. However, detection of these peaks in the SOFAST-HMQC spectrum (Fig 5B) suggests that this experiment will be a useful method for recovering some backbone amide cross peaks that are broadened due to intermediate exchange with water (Yao et al., 2011).

Our expression system can be easily scaled up for production of larger quantities of ShK for *in vivo* studies. In addition, it offers an economical approach to producing isotopically-labelled peptides for NMR studies, thereby enabling studies of the dynamics of ShK in solution and of its interaction with Kv1.3 channels. ^{15}N - and/or ^{13}C -labelled ShK can be used for NMR studies of ShK binding to Kv1.3-KcsA chimeras reconstituted in detergent micelles (Chill et al., 2006; Imai et al., 2010) or lipid bilayers (Lange et al., 2006). The

results from such studies, in conjunction with site-directed mutagenesis, will be invaluable in refining our understanding of the interaction of ShK with Kv1.3 leading to the design of ShK analogues with increased specificity for Kv1.3 channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

T_{EM}	effector memory T-cells
K_v	voltage-gated K ⁺ channel
KCa	Ca ²⁺ -activated K ⁺ channel
ShK	K ⁺ channel toxin from <i>Stichodactyla helianthus</i>
Trx-ShK	ShK fused with thioredoxin solubility tag
HSQC	heteronuclear single quantum coherence

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Highlights

- ShK is a potent potassium channel blocker
- A recombinant expression system for ShK has been established
- Recombinant ShK is equipotent with synthetic ShK in a range of bioassays
- Expression yield is maintained in minimal media suitable for isotopic labelling

Fig. 1. Structure of ShK. (A) ShK sequence showing its three disulfide bonds: Cys3 to Cys35, Cys12 to Cys28 and Cys17 to Cys32. (B) Ribbon view of the solution structure of ShK (PDB ID: 1ROO) where the disulfide bonds are coloured grey. (C) Surface representation of ShK structure. Residues crucial for K^+ -channel binding include Lys22 (blue) and Tyr23 (purple). For colour references, please refer to the online version.

Fig. 2.

Expression and purification of Trx-ShK. (A) Schematic of thioredoxin-ShK fusion protein. The N-terminal thioredoxin (red) and C-terminal ShK (yellow) proteins were fused by a polypeptide linker containing a hexa-His Tag (blue) and an enterokinase recognition site (light green). (B) SDS-PAGE of extracts from the lysed, uninduced and IPTG-induced *E. coli* cells were visualized by Coomassie brilliant blue staining; lane 1, uninduced cells; lane 2, *E. coli* extract of induced cultures (Trx-ShK is indicated by an arrow). (C) Solubility analysis of *E. coli* lysate; lane 1, soluble cell extract; lane 2, insoluble pellet. (D) Cleavage of thioredoxin fusion protein with enterokinase; lane 1, NTA purified refolded thioredoxin-ShK fusion protein; lane 2, Cleaved fusion protein (ShK migrates at approximately 4 kDa; indicated by an arrow with a dashed line).

Fig. 3. Characterisation of recombinant ShK. (A) RP-HPLC chromatogram where peaks 1 and 2 correspond to recombinant ShK and the cleaved thioredoxin fusion partner, respectively. The right axis represents the percentage of acetonitrile. (B) Analytical RP-HPLC chromatograph of purified recombinant ShK. (C) High-resolution ESI-TOF analysis of unlabelled recombinant ShK. Mass spectra were analyzed using Masslynx software (version 4.1), which gave a mass of 4055 Da for recombinant ShK.

Fig. 4. 1D ¹H NMR spectra (600 MHz) of recombinant unlabelled ShK (top, in black) and synthetic ShK (bottom, in red). The samples were prepared in 90 % H₂O/10 % ²H₂O at pH 4.9, 293 K. The residual water resonance between 4.75 ppm and 5.25 ppm was replaced with a dashed line for clarity. The ShK spectra are identical except for traces of impurities, eg. the ammonium peaks around 6.5 ppm in the spectrum of recombinant ShK.

Fig. 5.

(A) [^1H - ^{15}N] HSQC spectrum of ^{15}N -labelled ShK recorded in 90 % H_2O /10 % $^2\text{H}_2\text{O}$ at pH 4.9 and 293 K. Lys and Arg side chain peaks, which were folded from outside the acquired ^{15}N spectral width, are highlighted by a box. The amide side chain peaks of Gln16 at 6.25/108 and 6.35/108 ppm are joined by a line. (B) SOFAST-HMQC spectrum of the same sample, showing recovery of the Cys3 and Met21 backbone amide cross peaks (Yao et al., 2011).

Fig. 6.

Recombinant ShK exhibits a high affinity for Kv1.3 and Kv1.1 K^+ channels. (A) Effect of recombinant ShK (rShK) on mKv1.3 currents in stably transfected L929 cells. (B) Effect of synthetic ShK (sShK) on mKv1.1 currents in stably transfected L929 cells. (C) Dose-dependent inhibition of mKv1.3 (○, K_d 25 ± 10 pM) and mKv1.1 (Δ, K_d 15 ± 5 pM) currents by recombinant ShK. Each data point is the mean of 3–5 determinations. (D) Dose-dependent inhibition of mKv1.3 (○, K_d 10 ± 2 pM) and mKv1.1 (Δ, K_d 21 ± 3 pM) currents by synthetic ShK. Each data point is the mean of 3–5 determinations. (E) Selectivity of synthetic ShK and recombinant ShK for mKv1.3 and mKv1.1 channels. (F) Recombinant ShK and ShK-186 inhibit the proliferation of human peripheral blood lymphocytes, as

determined by [^3H] thymidine incorporation (rShK: ▼, IC_{50} 2.2 ± 0.4 nM; ShK-186: *, IC_{50} 4.1 ± 0.4 nM). (G) Recombinant ShK preferentially inhibits the proliferation of rat T_{EM} cells (■, IC_{50} 738 ± 89 pM) with minimal inhibition of naïve/ T_{CM} lymphocytes (○).