Expression in *Pichia pastoris* and characterization of APETx2, a specific inhibitor of acid sensing ion channel 3

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**A B S T R A C T**

Acid sensing ion channels (ASICs) are family of proteins predominantly present in the central and peripheral nervous system. They are known to play important roles in the pathophysiology of pain and ischemic stroke. APETx2 is a potent and selective inhibitor of ASIC3-containing channels and was isolated from sea anemone *Anthopleura elegantissima*. To facilitate the study on the molecular determinants of ASIC3-ligand interactions, we expressed recombinant APETx2 in the *Pichia pastoris* (*P. pastoris*) expression system and purified it to homogeneity. Recombinant APETx2 produced in *P. pastoris* inhibited the acid-evoked ASIC3 current with the IC50 value of 37.3 nM. The potency of recombinant toxin is similar to that of native APETx2. The sequential assignment and structure analysis of APETx2 were obtained by 2D and 3D 15N-edited NMR spectra. Our NMR data suggests that APETx2 produced in *P. pastoris* retained its native fold. The results presented here provide the first direct evidence that highly disulfide bonded peptide inhibitor of ASIC3, APETx2, can be expressed in *P. pastoris* with correct fold and high yield. We also showed that the R17A mutant exhibited a decrease in activity, suggesting the feasibility of the use of this expression system to study the interactions between APETx2 and ASIC3. These evidences may serve as the basis for understanding the selectivity and activity of APETx2.

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1. Introduction

Acid-sensing ion channels (ASICs) are proton-gated cationic channels expressed primarily in peripheral sensory neurons and in the neurons of the central nervous system. Proton activation of ASICs is important in a variety of physiological and pathological processes, such as nociception, mechanosensation, synaptic plasticity, and acidosis-mediated neuronal injury (Drew et al., 2004; Hildebrand et al., 2004; Roza et al., 2004). The ASIC protein family contains seven subtypes: ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC 4, and ASIC5. They form either a homomeric or a heteromeric functional channel. Functional trimeric ASICs are activated by external pH variations ranging from pH 6.8 to 4.0, and the ASICs-mediated currents are characterized by either rapid kinetics of inactivation (ASIC1a, ASIC1b, ASIC3) or slow kinetics of inactivation (ASIC2a) (Lingueglia, 2007). ASIC3 is expressed predominantly in dorsal roots and trigeminal ganglia as well as in the sensory nerve endings of the skin (Price et al., 2001). ASIC3 is involved in response to heat, acid, and...
mechanical stimuli, and mediates pain sensation during myocardial ischemia (Benson et al., 1999; Sutherland et al., 2001). ASIC3 knockout mice are more sensitive to light touches and less sensitive to noxious pinches than normal mice are, and that they are desensitized to intramuscular acid injections (Price et al., 2001; Chen et al., 2002; Sluka et al., 2003). Therefore, understanding the interaction between ASIC3 and its ligands is important for understanding their physiopathological function.

The problem of finding selective antagonists for studying ion channels has been largely solved by studying animal venoms, which offer many peptide toxins with an immense variety of pharmacological properties (Harvey et al., 1994; Tytgat et al., 1999; Escoubas et al., 2000b; Shakkottai et al., 2001; Bosmans and Tytgat, 2007). Animal venom toxins are rich sources of potent and selective antagonists for ASICs (Escoubas et al., 2000b; Diochot et al., 2004). For example, PcTx1 and APETx2, isolated from spiders and sea anemones, are selective and high-affinity inhibitors of ASIC1a and ASIC3-containing channels, respectively (Escoubas et al., 2000b; Diochot et al., 2004). PcTx1 specifically inhibits ASIC1a homomers with an IC50 value of 0.7 nM. Because PcTx1 does not inhibit ASIC1a-containing heteromers, it is used to characterize ASIC1a homomeric channels in peripheral and central nervous system neurons (Escoubas et al., 2000a). It is a gating modifier and shifts the channel from a resting to an inactivated state by increasing its affinity for protons. APETx2, a 42-amino acid peptide toxin isolated from the sea anemone Anthopleura elegantissima, is a potent and selective inhibitor of homomeric ASIC3 and ASIC3-containing channels. It reduces transient peak acid-evoked currents mediated by homomeric ASIC3 channels in heterologous expression systems and in primary cultures of sensory neurons (Diochot et al., 2004; Chen et al., 2005).

APETx2 inhibits rat and human homomeric ASIC3 channels with the IC50 values of 63 nM and 175 nM, respectively. It also inhibits heteromeric ASIC3/1a, ASIC1/3b, and ASIC3/2b with the IC50 values of 2 μM, 900 nM, and 117 nM. In contrast, homomeric ASIC1a, ASIC1b, and ASIC2a, and heteromeric ASIC3/2a channels are not sensitive to APETx2 (Diochot et al., 2004). Both PcTx1 and APETx2 are cross-linked by three disulfide bonds (Chagot et al., 2005; Escoubas et al., 2003). However, they have very low sequence homology. The mode of action for APETx2 is unknown. Similar to other sea anemone peptides, APETx2 may also act as a specific gating modifier for ASIC3. Because the activation of ASIC3 has been implicated in inflammatory and ischemic pain, APETx2 may be a potential analgesic agent (Price et al., 2001; Chen et al., 2002; Sluka et al., 2003; Deval et al., 2008; Yagi et al., 2006).

Site-directed mutagenesis on the residues of toxins is a useful tool for identifying their interactions with their receptors. Because most peptide toxins contain many disulfide linkages, it is difficult to express them with the correct fold. In order to facilitate site-directed mutagenesis of APETx2 for functional analysis, to study the structure–function relationships of ASIC3 and APETx2, and to understand the molecular mechanisms that lead to the inhibition of ASIC3 by APETx2, it is essential to express and label the protein with the correct fold and with a high yield.

We recently have expressed highly disulfide-bonded snake venom toxins in Pichia pastoris (P. pastoris) with the correct fold and with high yields (Guo et al., 2001; Shiu et al., 2004; Chen et al., 2006; Anangi et al., 2007). Therefore, we were able to synthesize the APETx2 gene and express it in P. pastoris. We here report that APETx2 expressed in P. pastoris has the same function and structure as native protein, and that the yield of APETx2 produced from P. pastoris is 2–4 mg/L after purification. We also found that R17A mutant activity decreased, which suggests that using this expression system for site-directed mutagenesis on APETx2 is feasible. Our findings may provide a basis for exploring the interactions between APETx2 and ASIC3.

2. Materials and methods

2.1. APETx2 expression in P. pastoris and purification

APETx2 was expressed in P. pastoris using yeast transfer vector, pPICZα A and a kit (EasySelect Expression Kit, version A; Invitrogen Corp., Carlsbad, CA, USA) with minor modifications of the manufacturer’s protocols. A synthetic gene composed of codons preferentially used in P. pastoris and coded for APETx2 was made using an overlapping oligonucleotide strategy and a polymerase chain reaction (PCR). The sense primer 5′-GAATTCGAATTCCATCATCATCATCATGTAAGGAAATGTGCTTTCTC-3′ contained Eco RI recognition and six histidine residues for purification. The antisense primer was 5′-GCGGCCGCGGTCAGTGTCATGTCATCAGTGTAAAGAGAATGTGCTTTCTC-3′ with Sac II recognition and a TCA stop-codon. The PCR product was purified and then cloned into the Eco RI and Sac II sites of pPICZα A. The recombinant plasmid was transformed into the DH5α strain, and the colony was selected using an agar plate with low-salt LB medium (1% tryptone + 0.5% yeast extract + 0.5% NaCl + 1.5% agar [pH 7.5]) and 25 μg/ml of zeocin antibiotic. After the clone had been confirmed by sequencing the insert, 10 mg of plasmid was digested with Sac I to linearize the plasmid. The linearized construct was transformed into Pichia strain, X-33, using the heat-shock method, and the transformation was done using a kit (Pichia EasyComp; Invitrogen). The transformant was integrated at the 5′ AOX1 locus using a single crossover, and the colony was selected using an agar plate with yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and 100 μg/ml of zeocin. PCR analysis was used to analyze Pichia integrants to determine whether the APETx2 gene had integrated into the Pichia genome, and then the cells were lysed (Lyticase; Sigma–Aldrich Co., St Louis, MO, USA). We picked the highest APETx2 protein expression clone from a number of clones with multiple copies of APETx2 gene insertion.

The unlabeled APETx2 was produced as follows: a 100 μl of cells stock grew at 30 °C in 100 ml of YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) containing with 100 μg/ml of zeocin for 48 h. Cells were then transferred into 900 ml of YPD medium. After another 48 h, the cells were collected by centrifugation and grown in 1 L of minimal methanol medium (1.34% yeast nitrogen base (YNB) with ammonium sulfate but without amino acids),
and with $4 \times 10^{-5}\%$ biotin. Methanol (1%) was added once every 24 h to induce protein APETx2 expression for 2 days.

The $^{15}$N-labeled APETx2 was produced as follows: 100 $\mu$l of cell stock was incubated at 30 °C in 100 ml of $^{15}$N minimal medium (0.34% YNB without ammonium sulfate or amino acids, but 2% dextrose and 0.05% $^{15}$NH$_4$Cl) in 100 mM of potassium phosphate buffer with 100 $\mu$g/ml of zeocin for 48 h. Cells were then transferred into 900 ml of $^{15}$N minimal medium. After another 24 h, the cells were centrifuged, collected, and then incubated in 1 L of $^{15}$N minimal medium in 100 mM of potassium phosphate buffer with $4 \times 10^{-5}\%$ biotin. Methanol (1%) was maintained to induce APETx2 expression for 48 h. The supernatant was centrifuged, collected, and then dialyzed twice against 10 L of H$_2$O and once against 5 L of binding buffer (50 mM of phosphate buffer [pH 8.0]). The dialyzed solution was loaded into an Ni$^{2+}$-chelating column, and proteins were eluted using a buffer containing 250 mM of imidazole. The final solution was loaded into an SP-Sepharose cation exchange column and eluted with a gradient of 500 mM of NaCl [pH 6.5]. Proteins were further purified using C18 reverse phase HPLC with a gradient of 20–30% acetonitrile. The recombinant APETx2 was more than 95% pure, as determined using tricine–SDS-PAGE (Schagger and von Jagow, 1987).

2.2. Mass spectrometric measurements

The molecular weights of proteins were confirmed using a triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo ion spray (TurbolonSpray; PE Sciex, Thornhill, ON, Canada). Protein solutions (1–10 $\mu$M in 50–90% methanol or acetonitrile with 0.1% formic acid) were infused into the mass spectrometer using a syringe pump (Harvard Apparatus, South Natick, MA, USA) at a flow rate of 12–20 $\mu$L/min to acquire full-scan mass spectra. The electrospray voltage at the spraying needle was optimized at 5000–5300 V. The molecular weights of proteins were calculated using software provided with the spectrometer.

2.3. N-Terminal sequence analysis

Recombinant APETx2 was analyzed using tricine–SDS-PAGE and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was stained with 0.2%...
amidoblack. The bands were excised and analyzed using a sequencer (model 477A; Applied Biosystems).

2.4. Molecular cloning and heterologous expression of ASIC3

Mouse acid-sensing ion channel 3 (mASIC3) was cloned from mouse DRG cDNA using a PCR. PCR involved a primer 68 bp upstream of the start codon (5’-GGCCTCCTGAATCTTATCTT-3’) and a primer downstream of the stop codon (5’-TAGGACTTTATTTGGGGTGAA-3’) using TaqDNA polymerase with 1 M of betaine (Invitrogen). After they had been subcloned into pBluescript (pBS) II vector (Promega, Madison, WI, USA), the clones were sequenced on both strands. mASIC3 was then subcloned to pIRES-hrGFP-2a vector (Stratagene, La Jolla, CA, USA). Lipofectamine (Invitrogen) was used to transfect the pIRES-hrGFP-2a vector with the recombinant mASIC3 into CHO-K1 (Chinese Hamster Ovary K1) cells. Whole-cell patch recording was done the next day.

2.5. Preparation of DRG primary cultures

Acid-sensing ion channel 3 knockout (ASIC3−/−) mice were generated and backcrossed with the CD1 wild-type (ASIC3+/+) mice as previously described (Chen et al., 2002; Lin et al., 2008). Adult ASIC3+/+ and ASIC3−/− mice (8–12 weeks old) were used for DRG primary culture (Chen et al., 2002; Lin et al., 2008). All procedures followed the Guide for the Use of Laboratory Animals (National Academy Press, Washington, DC, USA) and were approved by the Institutional Animal Care and Use Committee of Taiwan’s Academia Sinica.

2.6. Whole-cell patch clamp recording

Patch-clamp recordings were done as described elsewhere (Lin et al., 2008).

2.7. Drugs and solutions

A 100% ethanol-based 1-M solution of salicylic acid (SA) was turned into a 500-μM solution in artificial cerebrospinal fluid (ACSF) with a final ethanol concentration of <0.1%. The ACSF [pH 5.0] was then titrated using 2-[N-morpholino]ethanesulfonic acid (MES). The recombinant APETx2 and its R17A mutant was prepared with ACSF at the concentrations of 0.1, 1, 10, 100, and 1000 nM to obtain a dose-response curve and to calculate the IC50.

2.8. APETx2 inhibition on ASIC3-mediated current

The ACSF [pH 5.0] was applied for 4 s in 30 s intervals via a glass pipette 50 μm from the cells controlled by a VC-6 six channel valve controller (Warner Instruments, LLC, Hamden, CT, USA). SA (500 μM) or APETx2 peptides (0.1 nM–1000 nM) were used to determine whether the acid-evoked current
was blocked. The fitting equation for the dose-response curve of APETx2 inhibition was $y = A_2 + \frac{A_1}{(x/x_0)^p}$. $A_1$ represents the initial value, $A_2$, the final value, $x_0$, the center (point of inflection), and $p$, the power that affects the slope of the area around the inflection point. We used 100% as the maximal response to fit the data.

2.9. NMR spectroscopy

All 2D/3D NMR spectra were obtained at 600 MHz using an NMR spectrometer (Avance 600; Bruker BioSpin Corporation, Billerica, MA, USA). Samples were dissolved in 10% D$_2$O/90% H$_2$O or 100% D$_2$O at a concentration of 2.0 mM, the pHs were adjusted with 100 mM KOD to 3.0. The data were processed on the SGI O2 or Indigo 2 Extreme using XWINNMR software and analyzed using Aurelia software. 2D $^1$H NMR spectra were recorded in the phase-sensitive absorption mode with quadrature detection in both F1 and F2 dimensions (Marion and Wüthrich, 1983). A concentration of 3 mM $^{15}$N-labeled APETx2 was used for 2D $^1$H–$^{15}$N HSQC, 3D $^1$H–$^{15}$N-edited TOCSY-, and NOESY-HSQC experiments. Mixing times of 30–90 and 60–150 ms were used for TOCSY and NOESY experiments, respectively. The center frequencies of double resonance experiments were 4.75 ppm ($^1$H) and 118 ppm ($^{15}$N). The observed $^1$H chemical shifts were referenced with respect to the H$_2$O or HOD signal, which was taken as 4.75 ppm downfield from external sodium 3-trimethylsilylpropionate-2,2,3,3-d$_4$ (TSP) in D$_2$O (0.0 ppm) at 300 K. The nitrogen chemical shift was referenced to external $^{15}$NH$_4$Cl (3 mM in 1 M HCl) at 300 K, which is at 24.93 ppm downfield from liquid NH$_3$.

3. Results

3.1. Expression, purification and characterization of APETx2

APETx2 was expressed using pPICZa A vector in P. pastoris strain X-33. Recombinant APETx2 contained eight additional amino acid residues—EFHHHHHH—at the N-terminus. The EF residues were extra residues from the vector, and the six histidine tag residues were used for purification. Recombinant APETx2 produced in P. pastoris
was purified to homogeneity using Ni²⁺-chelating chromatography, SP-Sepharose cation exchange chromatography, and C18 reverse phase high performance liquid chromatography (HPLC) (Fig. 1). Mass spectrometry was used to determine its molecular weight. Mass analysis showed that recombinant APETx2 purified by Ni²⁺-chelating chromatography contained three major products, which were attributed to the partial cleavage produced by

![2D 1H-15N HSQC spectrum of 15N-labeled APETx2 produced in P. pastoris. Sample was recorded at 600 MHz at pH 3. Correlation peaks are labeled according to residues types and sequence numbers. The peaks connected by lines correspond to Gln and Asn side chain NH₂ groups.](image1)

![Disulfide patterns of APETx2. Aliphatic region of the NOESY spectra of APETx2 at pH 3 was shown and samples were dissolved in 100% D₂O. All six cysteine residues of APETx2 are identified by vertical broken lines.](image2)

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signal peptidases of *P. pastoris* (Supporting Information Fig. S1). We further separated them using SP-Sepharose cation exchange chromatography and HPLC (Fig. 1A and B). Based on N-terminal amino acid sequencing and mass spectrometry, Kex2 and Ste13 membrane-bound signal peptidases did not completely remove the EA repeats of recombinant APETx2. Peak I contained EAEA residues with an additional mass of 400.2 Da, and peak II contained EA residues with an additional mass of 200.1 Da. In contrast, peak III was fully cleaved by signal peptidases. Based on SDS-polyacrylamide gel electrophoresis, APETx2 produced in *P. pastoris* was purified to homogeneity (Fig. 1C). The final yields of unlabeled and $^{15}$N-labeled APETx2 were 2–4 mg/L and 0.5–1.0 mg/L, respectively. The experimental molecular

![Figure 6](image1.png)

**Fig. 6.** Amide strips from G1 to D42. They were taken from the 600 MHz 3D $^{15}$N-edited NOESY which illustrate the NOEs connectivity. The diagonal peaks are indicated by asterisks and both NH(i)–NH(i ± 1) and CaH(i)–NH(i ± 1) sequential NOEs are indicated by arrows.

![Figure 7](image2.png)

**Fig. 7.** Four-stranded $\beta$-sheet structure APETx2. The NOE connectivities and hydrogen bonds are shown in black arrows and dotted lines, respectively. Formation of hydrogen bonds was identified from 2D spectra recorded using 100% D$_2$O.

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weight of APETx2 produced in *P. pastoris* was 5660.4 Da, which was in excellent agreement with the calculated value of 5660.3 Da (Fig. 1D). The molecular weight of recombinant APETx2 included the additional 11712 Da of the eight extra amino acid residues (EHFHHHHHH) at the N-terminus. The mass of 5660.3 Da was calculated by assuming that all cysteines formed disulfide bonds, which indicated that three disulfide bonds had been formed in APETx2.

3.2. APETx2 inhibited ASIC3-mediated current

To test whether recombinant APETx2 selectively exhibits ASIC3, we examined the APETx2 inhibition of transient proton-gated current for salicylic acid-sensitive (SAS) DRG neurons, which contain ASIC3 ([Lin et al., 2008](#)). The APETx2 produced from *P. pastoris* blocked the transient proton-gated current in all tested SAS neurons (Fig. 2A). In contrast, APETx2 did not inhibit the transient proton-gated current in the SA-resistant transient (SART) neurons of ASIC3−/− mice (Fig. 2B). However, 1 μM of APETx2 inhibited transient proton-gated current in SAS neurons by only 50% (Fig. 2C). Therefore, we then used CHO cells transfected with ASIC3 to test whether APETx2 would inhibit ASIC3-mediated current. In CHO cells transfected with mouse ASIC3, the ASIC3-mediated current was triggered by a drop in pH from 7.4 to 5 (Supporting Information Fig. S2). Both SA and APETx2 inhibited 90% of the ASIC3-mediated current (Fig. 3A). In contrast to wild-type APETx2, 1 μM of R17A mutant inhibited only 40% of the current (Fig. 3B and C). We further determined the IC50 value of APETx2 on CHO cells expressing ASIC3. APETx2 with an IC50 value of 37.3 nM inhibited ASIC3-mediated transient proton-gated current (Fig. 3D). However, the inhibition was incomplete, and 10% of the current remained unblocked. It is possible that the recombinant APETx2-insensitive current is the sustained ASIC current ([Diochot et al., 2004](#); [Yagi et al., 2006](#)). Recombinant APETx2 was stable and had a long shelf life at 4°C (data not shown).

3.3. NMR analysis of APETx2

NMR spectroscopy was used to examine the folding of APETx2 expressed in *P. pastoris*. Because 1H chemical shifts of APETx2 at pH 3 have been reported, we measured 2D and 3D NMR spectra of APETx2 under the same conditions (Figs. 4–6). Recombinant APETx2 showed that all 1H chemical shift values had deviated less than 0.1 ppm when compared with the reported values ([Chagot et al., 2005](#)). The well dispersed 1H–15N HSQC spectrum of the APETx2 produced in *P. pastoris* at pH 3 showed a similar pattern of NH chemical shifts, which indicated that the protein was correctly folded (Fig. 4).

Although the chemical shifts of the recombinant APETx2 produced in *P. pastoris* at pH 3 were consistent with those of native APETx2, it is still necessary to identify the disulfide pairings. We did a NOESY experiment using APETx2 at pH 3 in 100% D2O to determine the three disulfide bonds of APETx2. Their pairings can be determined by searching CbH to CbH, CzH to CbH, and CzH to CzH NOEs between different cysteines. Specifically, the NOEs between CbH and CbH of different cysteines are 98% unique ([Klaus et al., 1993](#)). Figs. 5 and 6 showed the NOESY spectra of APETx2 at pH 3 that NOE patterns of the disulfide bridges were examined. All three cysteine pairs (4–37, 6–30, and 20–38) were found from their CbH/CbH and CzH/CbH NOE patterns in the spectra (Fig. 5).

To examine the tertiary fold of the APETx2 produced in *P. pastoris*, we did heteronuclear 3D and homonuclear 2D NMR experiments using APETx2 at pH 3. The analysis of 3D structures of APETx1 and BDS-1, homologous proteins of APETx2, four-stranded β-sheet folds stabilized by three disulfide bridges. The formation of four-stranded anti-parallel β-sheets (residues 3–6, 9–15, 28–32, and 35–39) was characterized by the CzH–CzH, CzH–NH, and NH–NH NOE patterns of the connecting strands, and the slowly exchanging amide protons (Fig. 6). For example, strip plots of 15N-edited NOE of APETx2 clearly showed the NOEs between strand I and strand II (NH of C4 to CzH of I12, NH of C4 to NH of G11, and CzH of S5 to NH of G11), between strand II and strand IV (NH of W14 to CzH of G37, NH of W14 to NH of T36, and CzH of F15 to NH of T36), and between strand III and strand IV (NH of C30 to CzH of G38, NH of C30 to NH of C37, and CzH of R37 to NH of C37), which indicated the formation of four-stranded anti-parallel β-sheets (Fig. 7). Based on our NMR studies, recombinant APETx2 produced in *P. pastoris* maintained the same tertiary fold as native APETx2.

4. Discussion

In the present study, we expressed APETx2 in a *P. pastoris* expression system and protein was obtained using a three-step purification procedure with good yields. The recombinant APETx2 is as potent as that of native APETx2. We also found that (a) recombinant APETx2 retained the same folding as a native toxin, and (b) that its R17A mutant was less active, which suggested this expression system is feasible for studying the interactions between APETx2 and ASIC3.

Sea anemones are a rich source of peptide toxins that contain a variety of active peptides that function as modulators of voltage-gated Na+ and K+ channels, acid-sensing ion channels, or pore-forming blockers ([Honma and Shiomi, 2006](#)). Based on the three-dimensional fold and disulfide bond pattern of APETx2, it belongs to the β-defensin family, which includes anti-microbial peptides from humans, snakes, and sea anemones ([Torres and Kuchel, 2004](#)). In particular, APETx2 shares its sequence homology and three disulfide-bond patterns with many sea anemone peptide toxins, including Am-II, APETx1, BclV, BDS-I, and BDS-II ([Chagot et al., 2005](#)). This family of peptide toxins is classified as cysteine-rich all-beta toxins because of its conserved disulfide linkages, which are arranged in 1–5, 2–4, and 3–6 connections ([Diochot et al., 2007](#)). Despite their similar folds, this family of toxin peptides targets diverse ion channels. They have both a remarkable sequence homology and an equally notable variability in potency and selectivity in their interactions with their target receptors. For example, APETx2 shares 64% and 34% identity, and 76% and 57% homology with...
APETx1 and BDS-1, respectively (Chagot et al., 2005). However, both BDS-I and BDS-II target the voltage-gated Kv3.4 potassium channel, APETx1 targets human Ether-a-go-go (HERG) potassium channels, and APETx2 targets ASIC3 (Zhang et al., 2007). It appears that the differences in the interactions between toxins and channels are caused by different charge surface distributions of toxins (Chagot et al., 2005; Escoubas et al., 2003; Ferrat et al., 2001). It is also possible that the amino acid substitutions among them are responsible for their divergent targets. For example, it is hypothesized that two aromatic/basic clusters of amino acids of APETx2 are important for the interaction with ASIC3. One consists of the residues R17, R31, F15, Y16, Y32, and F33, and of the residues Y16, R17, P18, R31, and T36 (Chagot et al., 2005). The availability of recombinant APETx2 will be useful for testing this hypothesis and determining the recognition sequences for ASIC3 by mutating potential interacting region on APETx2. It was reported that APETx2 reversibly inhibited the rat and human ASIC3 peak currents with the IC50 values of 63 and 175 nM, respectively (Diochot et al., 2004). In our study, recombinant APETx2 expressed in P. pastoris inhibited ASIC3-mediated current in CHO-expressing mouse ASIC3 with an IC50 of 37.3 nM. Because the sequence of the mouse ASIC3 channel shares over 92% similarity with that of human and rat proteins, it is not surprising that the efficacy of recombinant APETx2 is similar to that of native toxin towards rat ASIC3 (Diochot et al., 2004; Hildebrand et al., 2004).

The E. coli expression system is not suitable for producing venom toxins with the correct fold, because most of them are highly disulfide-bonded (Anangi et al., 2007). In contrast, many proteins have been successfully expressed in eukaryotic systems (Anangi et al., 2007; Demain and Vaishnav, 2009). Even though eukaryotic expression systems allow the expression of proteins with the correct fold, they have certain disadvantages. For example, most proteins obtained from these expression systems are expensive, time-consuming to produce, and low-yield. In contrast, the methylotrophic yeast P. pastoris expression system combines several advantages of prokaryotic and eukaryotic expression systems (White et al., 1994). It is the only system that offers the benefits of E. coli (high-level expression, easy scale-up, and inexpensive growth medium) combined with the advantages of expression in a eukaryotic expression system (protein processing, folding, and post-translational modifications). To date, only an ASIC1a inhibitor, PcTx1, has been successfully produced in stable Drosophila melanogaster S2 cells with a yield of 0.5 mg/L (Escoubas et al., 2003). However, the cost of insect-cell medium is very high. A refolding procedure was optimized for APETx2, and the yield was 45%. To reduce the cost of protein production and to avoid the need of refolding, we expressed APETx2 in P. pastoris. The sequential assignment and structural analysis of APETx2 were done using 2D and 3D 15N-edited NMR spectra. Our analysis of the NOE patterns between different cysteines, the chemical shifts, and secondary structures of APETx2 indicate that the folding of the toxin produced in P. pastoris was correct. The disulfide pairings of APETx2 were in close proximity, which is consistent with the reported disulfide pairings (Chagot et al., 2005). The present study showed that highly disulfide-bonded sea anemone toxins can be expressed with the correct fold and high yield. To study the detailed interactions between APETx2 with ASIC3, we used the P. pastoris expression system to produce APETx2 mutant proteins. Our preliminary NMR results showed that they retained the same folding as native protein. Our present study may serve as the basis for uncovering the structure–function relationships of ASIC3 and its ligands and for understanding diverse functions of this protein fold.

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Conflict of interest

None declared.

Appendix

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxicon.2010.08.004.

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