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Refolding of disulfide containing peptides in fusion with thioredoxin

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Materials and Methods

Gene Synthesis

The DNA sequence encoding peptide Ueq12-1 was constructed by PCR technique using synthetic oligonucleotides as described.[1] The DNA sequence encoding peptide APETx2 was constructed by PCR technique using synthetic oligonucleotides A2-dir1 (CTC CTT AGA TCT GAT GGG TAC CGC TTG CTC TTG CG) containing the Met codon for CNBr cleavage, A2-dir2 (TCC AAA GGC ATC TAC TGG TTC TAC CGT CCG TCT TGC CCT ACC GAC CGT), A2-dir3 (GGT TAC ACC GGT TCT TGC CGT TAC TTC CTT GGT ACC TGC TGC ACT CCG), A2-rev1 (CCA GTA GAT GCC TTT GGA GTT ACC GCA AGA GCA AGC), A2-rev2 (GCA AGA ACC GGT GTA ACC ACG GTC GGT AGG GCA AGA) and A2-rev3 (GGA ATT CCT AGT CAG CCG GAG TGC AGC AGG TAC CA). The amplified PCR fragments were gel-purified and cloned into the expression vector pET32b+ (Novagen).

Recombinant Peptides Production

Recombinant peptides produced as a thioredoxin fusion protein in *E. coli* BL21(DE3) or SHuffle. Cells were transformed with the expression vector and cultivated in LB medium with ampicillin (100 μg ml^{-1}) at 37°C. Expression of fusion proteins was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) up to 0.2 mM. After cultivation for 18 h at 25°C, cells were harvested by centrifugation (5 min at 6000×*g*), resuspended in a buffer for metal affinity chromatography (400 mM NaCl, 20 mM Tris-HCl, pH 7.5), and ultrasonicated. To remove all insoluble particles lysates were centrifuged for 15 min at 9000×*g*. Fusion proteins were purified using HisPur Ni-NTA metal affinity resin (Thermo Scientific, USA) using manufacturer protocols. To perform refolding the proteins were diluted to the concentration 1 mg ml^{-1} by water, GSH and GSSG were added to the solution up to 4 mM and 1 mM, correspondingly. Proteins solutions were held at 14ºC for 2–5 days. After that thioredoxin fusion proteins were cleaved in the dark at room temperature by CNBr with the addition of HCl up to 0.2 M, as described previously.[2] The target peptides were isolated from the reaction mixture using a reverse-phase column Jupiter C5 250×10 mm

(Phenomenex, USA). The purity of recombinant peptides was confirmed by N-terminal sequencing and ESI mass-spectrometry [Figure S1(*a*),(*b*)].

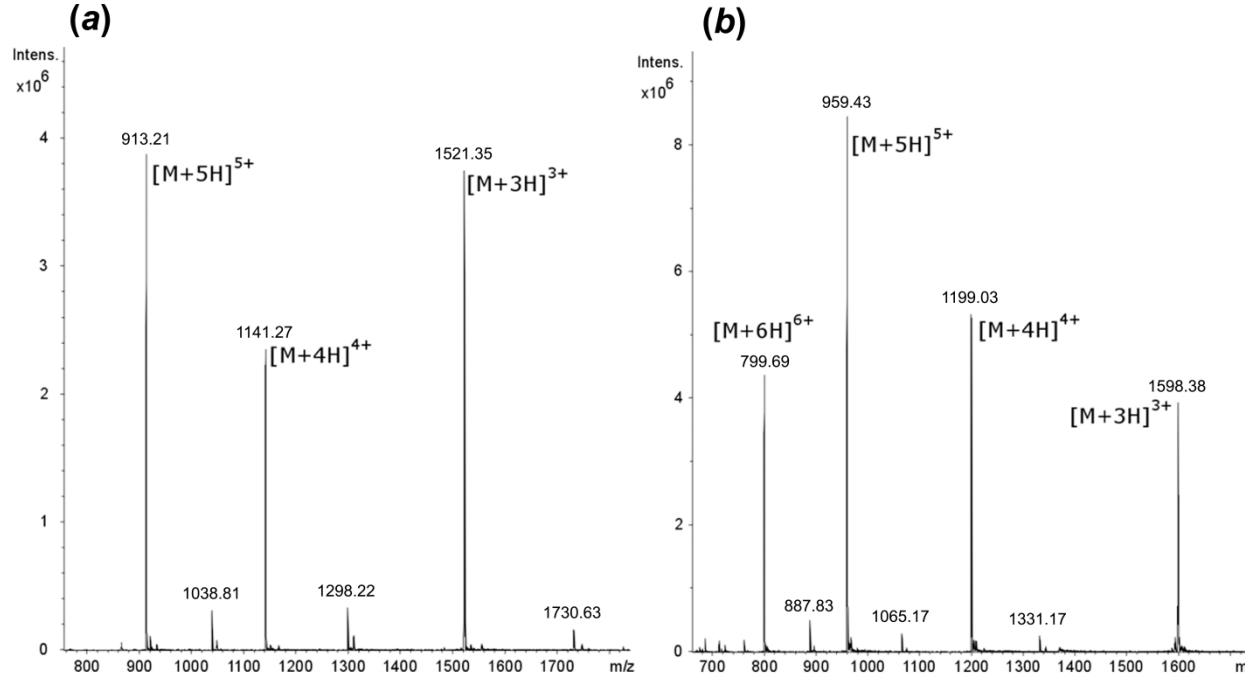

Figure S1 Data of mass-spectrometry analysis for recombinant peptides (*a*) APETx2 and (*b*) Ueq 12-1.

Electrophysiological Study on *Xenopus laevis* Oocytes

Oocytes expressed hASIC3 (Q9UHC3) homomeric channels and rat TRPA1 (Q6RI86) were prepared as described previously.[3,4] cRNA was synthesized from the plasmids using HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs Inc.) according to the manufacturer's protocol for capped transcripts. Intact oocytes were injected with 2–5 ng of cRNA transcript. Injected oocytes were incubated for 2–7 days at 15–19ºC in sterile ND-96 medium containing 100 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4, supplemented with 50 μg ml^{-1} gentamycin. Two electrode voltage clamp recordings were made using a GeneClamp500 amplifier (Axon Instruments) and microelectrodes filled with 3 M KCl solution. Data were filtered at 20 Hz and digitized at 100 Hz by an AD converter L780 (L-Card, Moscow, Russia) using homemade software. A computer-controlled valve system for a fast solution switch was used for ASIC3 activation. To induce ASIC3 currents, we employed ND-96-modified solutions in which 10 mM of HEPES was substituted for 5 mM MES pH 5.5. For study of Ueq 12-1 effects, oocytes injected with TRPA1 cRNA were clamped at −20 mV, recording of inward/outward currents made at repeated step to −100 mV for 80 ms following voltage ramp from −100 mV to +100 mV for 200 ms every 4 s. Diclofenac (300 μM in Ca^{2+}-free solution)[5] was used to activate the TRPA1 channel. Experiments were performed at room temperature (22–24°C).

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