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Effects of deletion and insertion of amino acids on the activity of HelaTx1, a scorpion toxin on potassium channels

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ABSTRACT

Four analogs of HelaTx1, a 25-mer peptide from scorpion venom, were synthesized by deleting its C-terminal hexapeptide fragment and N-terminal Ser residue and by inserting an amino acid in the middle part of the molecule. CD spectrum of HelaTx1(1−19) was almost superimposable to that of native HelaTx1. Functional characterization showed that HelaTx1(1−19) retained its inhibitory activity on Kv1.1 channel although 3 times less potent than HelaTx1, indicating that C-terminal part of HelaTx1 was not essential for its conformation and activity. Further deletion of N-terminal Ser residue and insertion of Ala in the middle part of the molecule affected the CD spectra and resulted in the decrease of activity.

1. Introduction

HelaTx1 1 is a peptide neurotoxin isolated from the venom of the Vietnamese forest scorpion *Heterometrus laoticus* (Fig. 1) (Vandendriessche et al., 2012). It consists of 25 amino acid residues with two disulfide bonds and belongs to the fifth subfamily member of the kappa scorpion toxins active on voltage-gated potassium channels (κ-KTx). From the channels studied, Kv1.1 and Kv1.6 were found to be the most sensitive. Competition experiments with tetraethylammonium showed that the toxin is a pore blocker. On the other hand, κ-hefutoxin 1 (HefuTx1) is the first toxin identified as κ-KTx family member and adopts a unique three-dimensional fold of two parallel helices linked by two disulfide bridges without any β-sheets (Srinivasan et al., 2002). Deletion of N-terminal three residues of HefuTx1 retained the conformation and activity. However additional deletion of a middle part residue between inner disulfide bonds (analog **M1**) resulted in the destruction of conformation and the loss of activity (Peigneur et al., 2013). Interestingly, HelaTx1 has less amino acid between inner disulfide bonds but shows activity.

In the present study, we synthesized four analogs of HelaTx1 (Fig. 1) with deletion of C-terminal six residues and N-terminal Ser residue and with insertion of Ala residue in the middle part of molecule in order to compare their CD spectra with that of HefuTx1. Inhibitory activity of HelaTx1 analogs on potassium channel Kv1.1 were also compared to that of native HelaTx1.

2. Materials and methods

2.1. Peptide synthesis

Solid phase peptide synthesis was performed on an Applied Biosystems 431A peptide synthesizer. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry was carried out with a PerSeptive Biosystems Voyager DE mass spectrometer using α-cyano-4-hydroxy-cinnamic acid as a matrix. Analytical and preparative HPLC were conducted on a Shimadzu LC-6A system with the ODS column Cosmosil $5C_{18}$-AR-II (4.6 × 250 mm, Nacalai tesuque) and Shimadzu LC-8A system with Cosmosil $5C_{18}$-AR-II (20 × 250 mm, Nacalai tesuque), respectively.

Abbreviations: CD, circular dichroism; Fmoc, 9-fluorenylmethoxycarbonyl; HefuTx1, κ-hefutoxin 1; MALDI-TOF-MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; ODS, octadecylsilane; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid.

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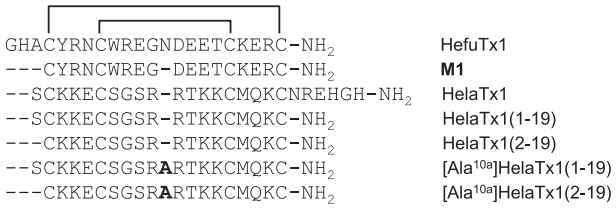

Fig. 1. Amino acid sequences of HelaTx1, HefuTx1 and their analogs.

For the synthesis of HelaTx1, a linear peptide was assembled on the resin by solid phase methodology of Fmoc chemistry in a 0.25-mmol scale. After TFA cleavage of a 0.1-mmol equivalent of the peptide resin, a linear peptide with free Cys residues was extracted with 2 M AcOH (200 ml). The solution was added to 1M NH_4OAc (1600 ml) containing GSSG (612 mg, 1.0 mmol), GSH (3.06 g, 10 mmol), and EDTA (584 mg, 2.0 mmol), and the solution was adjusted to pH 7.8 with aqueous NH_4OH and diluted to 2000 ml. The final concentration was 0.05 mM for peptide and 1 M for ammonium acetate buffer. The solution was stirred slowly at room temperature for three days to form the disulfide bonds. The crude oxidized peptide was purified by gel filtration (G-50F), ion exchange chromatography (CM-52), and preparative HPLC until they migrated as a single peak on analytical HPLC (yield, 45.6 mg; 11% from starting resin).

Four analogs of HelaTx1 were synthesized by the similar procedure although gel filtration was omitted for HelaTx1(2–19), [Ala^{10a}]HelaTx1(1–19), and [Ala^{10a}]HelaTx1(2–19). The yields were 28 mg (9%) for HelaTx1(1–19), 16.7 mg (5.5%) for HelaTx1(2–19), 44.1 mg in 0.075 mmol scale (19%) for [Ala^{10a}]HelaTx1(1–19), and 38.7 mg in 0.075 mmol scale (17%) for [Ala^{10a}]HelaTx1(2–19). The structures and purity of the synthetic peptides were confirmed by HPLC and MALDI-TOF-MS measurements.

2.2. Enzymatic digestion

Synthetic HelaTx1 (0.29 mg) and HelaTx1(1–19) (0.22 mg) were digested with trypsin (0.029 mg and 0.022 mg, respectively) in 0.1 M sodium phosphate buffer (0.5 ml, pH 6.5) at 37 °C for 2 h and subjected to HPLC separation. Molecular weights of enzymatic fragments were measured by MALDI-TOF-MS. Other three analogs were treated as described for HelaTx1(1–19).

2.3. CD measurements

CD spectra were recorded on a JASCO J-1100 spectropolarimeter in H_2O solution (10 mM sodium phosphate, pH 7.0) at 20 °C, with a quartz cell of 2-mm path length. The results are expressed as mean residue molar ellipticity $[\theta]_{MRW}$. Concentrations of the solutions were corrected by comparing the UV spectra and peak area on HPLC.

2.4. Expression in Xenopus oocytes

For the expression of the voltage-gated potassium channel $rK_V1.1$ in *X.* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The harvesting of stage V-VI oocytes from anaesthetized female *Xenopus laevis* frog was previously described (Liman et al., 1992). Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg/l gentamicin sulfate.

2.5. Electrophysiological recordings

Two-electrode voltage-clamp recordings were performed at room temperature (18–22 °C) using a Geneclamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data acquisition system (Axon Instruments, USA). Whole cell currents from oocytes were recorded 1–4 days after injection. Bath solution composition was ND96 (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.7 and 1.5 MΩ. The elicited currents were filtered at 1 kHz and sampled at 500 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. $K_V1.1$ current was evoked by 500 ms depolarizations to 0 mV followed by a 500 ms pulse to −50 mV, from a holding potential of −90 mV. In order to investigate the current–voltage relationship, current traces were evoked by 10 mV depolarization steps from a holding potential of −90 mV. All data represent at least 3 independent experiments ($n \geq 3$) and are presented as mean ± standard error.

3. Results

3.1. Peptide synthesis

Oxidative folding of linear precursors of HelaTx1 and its analogs showed single major peaks on analytical HPLC (Fig. 2). The crude oxidized products were purified by successive chromatography with three different mode of separation. MALDI-TOF-MS measurements gave $[M+H]^+$ 2917.28 for HelaTx1, 2186.39 for

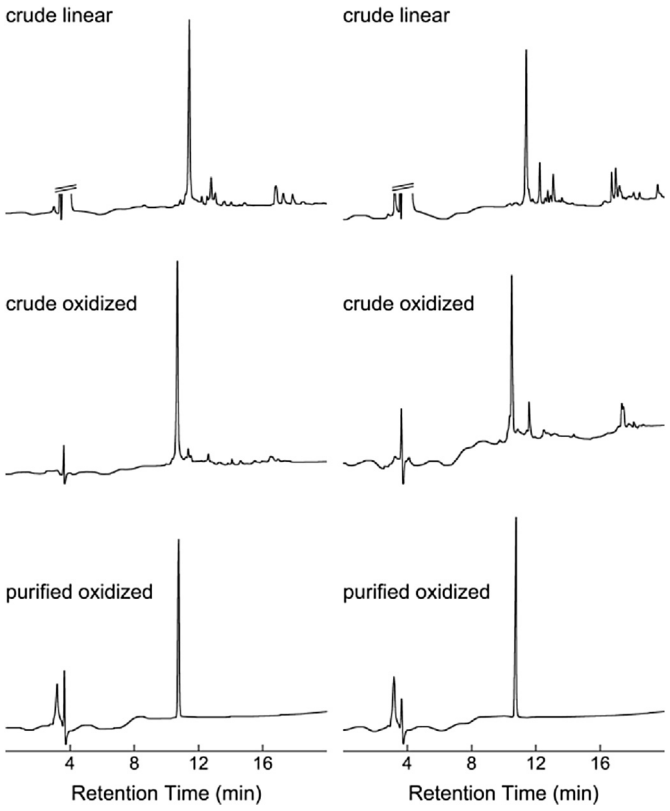

Fig. 2. Analytical HPLC profiles of synthesis of HelaTx1 (left) and HelaTx1(1–19) (right). Solvent: linear gradient from 5% to 65% CH_3CN in 0.1% TFA for 30 min. Flow rate: 1 ml/min. Monitoring: absorbance at 230 nm (intensities are not scaled).