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# Synthesis and characterization of amino acid deletion analogs of κ-hefutoxin 1, a scorpion toxin on potassium channels

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ABSTRACT

Nine analogs of scorpion toxin peptide κ-hefutoxin 1 were synthesized by stepwise deletion of its amino acid residues. Disulfide bond pairings of the synthetic analogs were confirmed by enzymatic digestion followed by MALDI-TOF-MS measurements. Functional characterization shows that analogs in which N-terminal residues were deleted retained biological activity, whereas deletion of middle part residues resulted in loss of activity. Furthermore, κ-hefutoxin 1 and analogs were subjected to a screening on voltage-gated potassium channels in order to determine their subtype selectivity. It is shown that κ-hefutoxin 1 is suitable as template for peptidomimetics in order to design small peptide-based therapeutic compounds.



## 1. Introduction

κ-Hefutoxin 1 is a peptide neurotoxin isolated from the venom of the Asian forest black scorpion *Heterometrus fulvipes* (Figs. 1 and 2) (Srinivasan et al., 2002). It adopts a unique three-dimensional fold of two parallel helices linked by two disulfide bridges without any β-sheets. Based on the presence of a functional diad ($Tyr^5$ and $Lys^{19}$) at a distance (6.0 ± 1.0 Å) comparable to other potassium channel toxins (Dauplais et al., 1997; Ranganathan et al., 1996; Savarin et al., 1998; Smith et al., 1997; Stampe et al., 1994), its function was hypothesized as a potassium channel toxin. κ-Hefutoxin 1 does indeed inhibit the voltage-gated potassium channels ($K_V$) $K_V1.3$ and $K_V1.2$. Moreover, it also slows the activation kinetics of $K_V1.3$ and is the first identified scorpion toxin capable of modifying the gating currents of $K_v$ channels. Mutation studies showed that a functional dyad composed of the residues $Tyr^5$ and $Lys^{19}$ is essential for the potassium current inhibiting activity (Srinivasan et al., 2002). κ-Hefutoxin 1 was the first family member of the kappa scorpion toxins active on voltage-gated potassium channels (κ-KTx) (Rodriguez de la Vega and Possani, 2004; Srinivasan et al., 2002). Up to date, this family is subdivided in 5 subfamilies and compromises more than 20 members (Camargos et al., 2011; Chen et al., 2012; Vandendriessche et al., 2012) (Fig. 1). Although not all κ-KTx have been functionally characterized, those who have been all show inhibiting activity on Kv1 channels except for κ-KTx1.3 (Chen et al., 2012; Nirthanan et al., 2005). It should be noted that κ-KTx are only active on $K_v1$ channels in higher micromolar concentrations, suggesting that these channels

*Abbreviations:* Acm, acetamidomethyl; CD, circular dichroism; Fmoc, 9-fluorenylmethoxycarbonyl; Hef-1, 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; Trt, triphenylmethyl.

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| | | |
|---|---|---|
| κ-KTx1.1 | κ-hefutoxin 1 | --GHA**C**YRN**C**WREGNDEET**C**KER**C**-------- |
| κ-KTx1.2 | κ-hefutoxin 2 | --GHA**C**YRN**C**WREGNDEET**C**KER**C**G------- |
| κ-KTx1.3 | κ-hefutoxin 3 | --GFG**C**YRS**C**WKAGHDEET**C**KKE**C**S------- |
| κ-KTx1.4 | HSP009C | --GFG**C**FRS**C**WKAGHDDKT**C**KSM**C**G------- |
| κ-KTx2.1 | OmTx1 | ---DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHPVE--- |
| κ-KTx2.2 | OmTx2 | ---DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHPVEY-- |
| κ-KTx2.3 | OmTx3 | --NDP**C**EEV**C**LQHTGNVKA**C**EEA**C**Q------- |
| κ-KTx2.4 | OmTx4 | ---DP**C**YEV**C**LQQHGNVKE**C**EEA**C**KHP----- |
| κ-KTx2.5 | OcyC8 | --YDA**C**VNA**C**LEHHPNVRE**C**EEA**C**KNPVPP-- |
| κ-KTx2.6 | OcyC9 | --FPP**C**VEV**C**VQHTGNVKE**C**EAA**C**GE------ |
| κ-KTx2.7 | HSP053C.1 | ---NA**C**IEV**C**LQHTGNPAE**C**DKA**C**DK------ |
| κ-KTx2.8 | HSP053C.2 | --GNA**C**IEV**C**LQHTGNPAE**C**DKP**C**DK------ |
| κ-KTx2.9 | HeTx204 | --GNA**C**IEV**C**LQHTGNPAE**C**DKA**C**DK------ |
| κ-KTx2.10 | HeTx203 | --GNA**C**IEV**C**LQHTGNPAE**C**DKP**C**DK------ |
| κ-KTx3.1 | HSP040C.1 | QWINA**C**FNV**C**MKISSDKKY**C**KYL**C**GKN----- |
| κ-KTx3.2 | HSP040C.3 | HWINA**C**FNI**C**MKISSDQKY**C**KSF**C**G------- |
| κ-KTx3.3 | HSP040C.4 | QWINA**C**FNI**C**MKISSDQKY**C**KSF**C**G------- |
| κ-KTx3.4 | HSP040C.5 | QWINA**C**FNV**C**MKISSDKKY**C**KYL**C**GKS----- |
| κ-KTx4.1 | HSP040C.2 | --DIP**C**FET**C**MKLYHIPKL**C**YIK**C**RKH----- |
| κ-KTx5.1 | HelaTX1 | ----S**C**KKE**C**SG-SRRTKK**C**MKQ**C**NREHGHGR |

**Fig. 1.** Multiple sequence alignment of κ-hefutoxin 1 with other existing κ-KTx family members.

are not the primary target of these toxins and, most likely they also act upon other yet unidentified targets. Interestingly, from cone snail species, 4 homologous peptides with the same conserved cysteine pattern have been isolated (Moller et al., 2005). Furthermore, peptides with a similar sequence but with an antimicrobial activity have also been identified in plants (Duvick et al., 1992). Altogether it can be concluded that the helix–loop–helix motif is conserved throughout different organisms. For peptides belonging to other structural families, such as ICK peptides, it has been shown that these peptides have followed a convergent as well a divergent evolution, resulting in a broad family with relatively diverse biochemical and biological functions (Zhu et al., 2003, 2005). Therefore, it can be believed that the extensive distribution of the helix–loop–helix common motif throughout diverse organisms highlights that this relatively stable and versatile scaffold has the potential to tolerate insertions, deletions and substitutions within the structure and thus represents an interesting template for peptide-based lead compounds in the development of novel medicines (Zhu et al., 2005).

In the present study, we synthesized a series of analogs of κ-hefutoxin 1 in which N-terminal and middle part

| | |
|---|---|
| GHACYRNCWREGNDEETCKERC-$NH_2$ | Hef-1 |
| -HACYRNCWREGNDEETCKERC-$NH_2$ | **N1** |
| --ACYRNCWREGNDEETCKERC-$NH_2$ | **N2** |
| ---CYRNCWREGNDEETCKERC-$NH_2$ | **N3** |
| ---CYRNCWREG-DEETCKERC-$NH_2$ | **M1** |
| ---CYRNCWRE--DEETCKERC-$NH_2$ | **M2** |
| ---CYRNCWRE---EETCKERC-$NH_2$ | **M3** |
| ---CYRNCWR----EETCKERC-$NH_2$ | **M4** |
| ---CYRNCWR-----ETCKERC-$NH_2$ | **M5** |
| ---CYRNCWR------TCKERC-$NH_2$ | **M6** |

**Fig. 2.** Amino acid sequences of κ-hefutoxin 1 and its analogs.

amino acids were deleted in order to study the influence of such structure modifications on the activity and selectivity profile of this toxin and herewith gain more insight in the structure–function properties of this peptide as a template of drug design (Fig. 2).

## 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. LC-MS was measured on a ThermoFisher LTQ-Orbitrap with Shiseido Nanospace SI-2. Analytical and preparative HPLC were conducted on a Shimadzu LC-6A with the ODS columns Shim-pack CLC-ODS (4.6 × 250 mm, Shimadzu) and Shimadzu LC-8A system with Shim-pack PREP-ODS (H) (20 × 250 mm, Shimadzu), respectively.

First we used simple single-step random air oxidation strategy for the synthesis of all analogs as described for the synthesis of κ-hefutoxin 1 (Srinivasan et al., 2002) (Fig. 3). However, synthesis of **M6** was unsuccessful due to dominant formation of a dimeric product. Therefore, standard compound of **M6** was synthesized by selective two-steps disulfide bond formation method in which Trt and Acm group were used for the protection of Cys residues (Fig. 3) (Balaji et al., 2000). For the synthesis of standard **M6**, a linear peptide containing Cys(Acm) at the 1st and 13th positions 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 at the 5th and 9th



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