**Active Sites of Spinoxin, a Potassium Channel Scorpion Toxin, Elucidated by Systematic
Alanine Scanning**

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ABBREVIATIONS

EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; KTx, K⁺-channel-specific scorpion toxins; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MTX, maurotoxin; Pi1 and Pi4, potassium channel-blocking toxin 1 and 4; RP-HPLC, reversed phase high performance liquid chromatography; SPX, spinoxin; TFA, trifluoroacetic acid

Analogs are designated by a letter and number indicating the identity and position of the substituted amino acid, followed by a letter indicating the identity of the replacement residue. For example, **K23A** indicates an analog in which Lys²³ is replaced with Ala.

ABSTRACT:

Peptide toxins from scorpion venoms constitute the largest group of toxins that target voltage-gated potassium channel (Kv). Spinoxin (SPX) isolated from the venom of scorpion *Heterometrus spinifer*, is a 34-residue peptide neurotoxin cross-linked by four disulfide bridges. SPX is a potent inhibitor of Kv1.3 potassium channels ($IC_{50} = 63$ nM), which are considered to be valid molecular targets in the diagnostics and therapy of various autoimmune disorders and cancers. Here we synthesized 25 analogs of SPX and analyzed the role of each amino acid in SPX using alanine scanning to study its structure-function relationships. All synthetic analogs showed similar disulfide bond pairings and secondary structures as native SPX. Alanine replacements at Lys²³, Asn²⁶, and Lys³⁰ resulted in loss of activity against Kv1.3 potassium channels, whereas replacements at Arg⁷, Met¹⁴, Lys²⁷, and Tyr³² also largely reduced inhibitory activity. These results suggest that the side chains of these amino acids in SPX play an important role in its interaction with Kv1.3 channels. In particular, Lys²³ appears to be a key residue that underpins Kv1.3 channel inhibition. Of these seven amino acid residues, four are basic amino acids, suggesting that the positive electrostatic potential on the surface of SPX is likely required for high affinity interaction with Kv1.3 channels. This study provides insight into the structure-function relationships of SPX with implications for the rational design of new lead compounds targeting potassium channels with high potency.

Voltage-gated potassium ion channels (Kv) are present in a wide variety of cells and play a key role in electrical excitability, cell proliferation, apoptosis and volume regulation.¹ Of considerable interest was the demonstration that a subset of T lymphocytes (autoreactive memory T lymphocytes), considered to be major mediators of autoimmunity, expressed large numbers of Kv1.3 channels,² opening the possibility that Kv1.3 channels could potentially be valid targets for the therapeutic modulation or diagnosis of autoimmune disorders involving these lymphocyte subsets.^{2,3} Furthermore, Kv1.3 channels have also been considered as a potential molecular target for the diagnostics and therapy of some cancers.^{1,4}

Several scorpion toxins (KTx) targeting Kv channels have been reported so far, with diverse pharmacological selectivity for various subtypes of Kv channels. These toxins consist of 23–43 amino acid residues and are classified into four subfamilies,^{5,6} of which α -KTx is the largest subfamily that shares a common cysteine-stabilized α/β motif (C $\alpha\beta$).

We previously isolated and characterized spinoxin (SPX) from the venom of Malaysian black scorpion *Heterometrus spinifer* (Scorpionidae). SPX is a 34-residue peptide with four disulfide bridges and belongs to α -KTx6 subfamily.⁷ We also have identified that the disulfide bond pairings of SPX are Cys1–Cys5, Cys2–Cys6, Cys3–Cys7, and Cys4–Cys8, which is commonly found in α -KTxs.⁸ SPX inhibits Kv1.2 and Kv1.3 channels, but has no inhibition activity against Kv1.1 channels (Table 1). SPX shares high amino acid sequence identity with Pi4 (85%) from *Pandinus imperator*⁹ and maurotoxin (MTX) (82%) from *Scorpio maurus palmatus*.¹⁰ However, unlike SPX, Pi4 has no inhibition activity against Kv1.3⁹ and MTX inhibits Kv1.1 (Table 1),¹¹ indicating that the structure of SPX is probably different from that of Pi4 and MTX. Furthermore, α -KTxs such as Pi4 and MTX have been studied extensively,^{12–15} whereas the structure-function relationships of SPX are poorly understood.

In this study, to investigate the structure-function relationships of SPX, we first synthesized 25 analogs of SPX in which each amino acid residue (except for cysteine) was systematically replaced by alanine (i.e. comprehensive alanine scanning), and analyzed the disulfide bond pairing and secondary structure of each analog. Next, to evaluate the pharmacological activity against Kv channels, we used Kv1.3 channels as targets of SPX, because Kv1.3 has recently

1 been reported to be related to autoimmune disorders and several types of cancers,^{16, 17}
2 although SPX is approximately 25-fold selective for Kv1.2 over Kv1.3 (Table 1). Thus, we
3 have identified seven key amino acid residues in SPX for inhibition of Kv1.3, of which the
4 four residues are located at less conserved positions in α -KTx6 subfamily. Comparison of
5 SPX with other scorpion toxins will provide new insights into the design of new therapeutic
6 products derived from scorpion toxins.

8 **EXPERIMENTAL PROCEDURES**

9 **Peptide synthesis.** Solid phase peptide synthesis was performed on an Applied Biosystems
10 431A peptide synthesizer (Applied Biosystems Inc., CA, USA). The analysis and the
11 purification of peptides was performed by RP-HPLC using an LC-6A system (Shimadzu,
12 Kyoto, Japan) with an ODS column Cosmosil 5C₁₈-AR-II (4.6 × 250 mm, Nacalai tesque,
13 Kyoto, Japan) and preparative HPLC was conducted using a Cosmosil 5C₁₈-AR-II column
14 (20 × 250 mm, Nacalai tesque, Kyoto, Japan). A linear precursor of **IIA**, whereby the first
15 residue Ile in SPX was substituted with Ala, was synthesized using solid phase methodology
16 with Fmoc chemistry starting from Rink amide resin (purchased from Applied Biosystems)
17 (0.25 mmol equivalent). In order to remove the protected groups and resin, the protected
18 peptide resin (583 mg, 0.083 mmol) was treated with TFA (8 mL) in the presence of
19 thioanisole (0.5 mL), H₂O (0.5 mL), phenol (0.75 mL), and 1,2-ethanedithiol (0.25 mL) at
20 0°C for 5 min and at room temperature for 1.5 h. After precipitation by addition of an excess
21 of diethyl ether, the crude linear peptide was collected by filtration and extracted with 2 M
22 AcOH (167 mL). Oxidation of the extracted peptide solution was performed by the addition
23 of 1 M NH₄OAc (1333 mL) with EDTA (487 mg, 1.67 mmol), GSSG (510 mg, 0.83 mmol),
24 and GSH (2.55 g, 8.33 mmol). The solution was adjusted to pH 7.8 with aqueous NH₄OH and
25 diluted to 1666 mL. The final concentration was 0.05 mM for the peptide and 1 M for
26 ammonium acetate buffer. The reaction solution was stirred slowly at 4°C for seven days to
27 form the disulfide bonds. The solution was loaded on to a PREP-ODS column (30 × 250 mm,
28 GL Sciences, Tokyo, Japan) and eluted with 50% CH₃CN in 0.1% TFA. The crude oxidized

product obtained after lyophilization of the solution was dissolved in 30% AcOH and applied onto a Sephadex G-50F column (5×107 cm) and eluted with the same solvent. Fractions containing the desired product were collected and lyophilized, then loaded onto a CM52 column (1.8×20 cm) and eluted using a linear gradient from 0.01 M NH_4OH (pH 4.5) to 0.7 M NH_4OH (pH 6.5). Fractions containing the desired product were collected and lyophilized and further purified by preparative RP-HPLC with an ODS column (20×250 mm) with isocratic mode. Purified **I1A** (yield, 35.6 mg; 9.6% from starting resin) showed a single peak on an analytical HPLC, with a linear gradient of solvent B (0.1% TFA/ CH_3CN) in solvent A (0.1% TFA/ H_2O), from 5–65% in 30 min at a flow rate of 1 mL/min. Other analogs were synthesized and purified using a similar procedure. The molecular mass of each synthetic peptide was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), carried out on a Voyager-DE PRO Biospectrometry Workstation (Applied Biosystems Inc., CA, USA) or an Autoflex III (Bruker Corp., Massachusetts, USA).

Enzymatic digestion. In order to determine the disulfide pairings, each synthesized analog was enzymatically digested with α -trypsin and α -chymotrypsin (bovine pancreas, Wako Pure Chemical Industries, LTD., Osaka, Japan). **I1A** (0.37 mg) was dissolved in 0.5 mL of 0.2 M Tris-HCl buffer (pH 7.1) containing 0.025 M CaCl_2 and digested with α -trypsin (37 μg) and α -chymotrypsin (37 μg) at 37°C for 23 hours. The digested peptide fragments were separated and collected by RP-HPLC with a linear gradient of solvent B (0.1% TFA/ CH_3CN) in solvent A (0.1% TFA/ H_2O), from 5–35% in 30 min at a flow rate of 1 mL/min. After lyophilization, the molecular mass of each fragment was measured by MALDI-TOF MS. Other analogs were treated the same as described above.

Expression in *Xenopus* oocytes. For the expression of the hKV1.3 channels in *Xenopus* oocytes, the plasmid pCI.neo containing the gene for Kv1.3 was linearized with NotI (New England Biolabs, USA) and was transcribed using the T7 mMESSAGE-mMACHINE

transcription kit (Ambion, USA). The harvesting of stage V-VI oocytes from anaesthetized female *Xenopus laevis* frog has been described previously.¹⁸ 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; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg/L gentamycin sulfate.

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; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4) or HK (in mM): NaCl, 2; KCl, 96; CaCl₂, 1.8; MgCl₂, 2 and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.8–1.5 MΩ. The elicited currents were filtered at 500 Hz and sampled at 1 kHz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. K_v1.3 currents were evoked by 500 ms depolarizations to 0 mV followed by a 500 ms pulse to -50 mV, from a holding potential of -90 mV. All data represent at least three independent experiments ($n \geq 3$) and are presented as mean $\pm$ standard error.

Circular dichroism (CD) measurements. The synthetic analog (0.1 μ mol) was dissolved into 2 mL of 0.01 M sodium phosphate buffer (pH 7.0) and recorded on a JASCO J-820 spectropolarimeter (Jasco, Tokyo, Japan) with a quartz cell of 1-mm path length at 20°C in the ranges from 250 nm to 190 nm. Data were collected at intervals of 0.2 nm with a scan rate of 10 nm/min and a time constant of 2 s ($n = 4$). The results are expressed as molar ellipticity $[\theta]$.

RESULTS

Peptide design and synthesis. To study the structure-function relationships of SPX, we first performed alanine scanning by systematically replacing each residue in the 34-residue

primary sequence of SPX with Ala, barring the Cys residues (Figure 1). Thus, we synthesized 25 analogs of SPX, in which each amino acid residue (except for eight Cys and one Ala in the native sequence) was replaced with Ala. The HPLC profiles of crude linear, crude and purified oxidized **I1A** are shown in Figure 2 as representative examples. All the analogs were synthesized using Rink amide resin, because SPX was amidated at the C-terminus. After TFA treatment, the peptide was precipitated with diethyl ether and extracted with 2 M AcOH. Oxidation of all the linear precursors to form disulfide bonds was performed by simple air-oxidation method with EDTA and GSH/GSSG. All the purified peptides showed a single peak using analytical HPLC. For each analog, the observed mass corresponded well with the theoretical mass of folded peptide (< 0.97 m/z).

Determination of disulfide bond pairing. In case of a peptide with multiple disulfide bonds, it is essential to show that the analogs have the same disulfide combinations as the native peptide. The disulfide bond pairings of each analog was determined by fragmentation with trypsin and chymotrypsin followed by the measurements of mass of each fragment. For example, **I1A** gave four fragments that matched with the calculated mass for the digested fragments QTGCPNGC-NH₂+H₂O, CSGSRCINK+H₂O, DCYSCK+H₂O, and SPCMKCY+H₂O (Figure 3A). α -Chymotrypsin cleaved at the C-terminus of Asn²¹ in SPX by non-specific digestion. These results showed that the disulfide bond of each fragment was Cys1–Cys5, Cys2–Cys6, Cys3–Cys7, and Cys4–Cys8, which corresponded to that of native SPX (Figure 3A, Table 2). The four analogs (**R7A**, **Y10A**, **K30A**, and **Y32A**) yielded fragments with two disulfide bonds, since the enzymatic cleavage sites were replaced with Ala (Figure 3B, Table 2). In order to determine the disulfide pairing of these four analogs, we designed two types of peptides with different disulfide bond pairing. One was a peptide that had the same disulfide bond pairing as that of SPX, and the other had a different disulfide bond pairing from that of SPX. To synthesize these two types of reference peptides, we used the two-step selective disulfide bond formation method¹⁹ with Trt (triphenylmethyl) and AcM (acetamidomethyl) protecting groups for cysteine residues (Figure 4A). For example, a

reference peptide **K30A-Fna** that has the same disulfide bond as SPX was synthesized from the precursor **K30A-Fna-P** with Fmoc chemistry starting from Fmoc-Lys(Boc)-Alko Resin (0.25 mmol equivalent) and used Trt protect group for Cys⁹ and Cys²⁹ and AcM protect group for Cys¹³ and Cys³¹ (numbering corresponding to **K30A**). To form the second disulfide bond, iodine oxidation was performed according to the method described previously.¹⁹ Purified **K30A-Fna-P** (0.4 mg) was digested with α -chymotrypsin (0.1 mg) at 37°C for 24 hours. **K30A-Fna** derived from **K30A-Fna-P** was separated and collected by RP-HPLC. After lyophilization, the molecular mass of each fragment was measured by MALDI-TOF MS, and matched with the theoretical mass of **K30A-Fna**. The other reference peptide **K30A-Fnon** with the non-native disulfide bond was also synthesized by the same method (Figure 4A, right). The fragment P3 obtained from **K30A** digestion (Figure 3B), **K30A-Fna**, and **K30A-Fnon** were analyzed using RP-HPLC with an isocratic elution mode (Figure 4B). The mixture solution of P3 and **K30A-Fna** was eluted as a single peak, but two peaks were detected for the mixture solution of P3 and **K30A-Fnon**, showing that **K30A** had the same disulfide bond pairings as native SPX. In the case of **R7A** and **Y10A**, similar results were obtained from experiments using the same method. The enzymatic digestion for **Y32A-Fnon-P** gave two fragments, because the C-terminus of Ala³² in **Y32A-Fnon-P** was cleaved by α -chymotrypsin. The mixture solution of the fragment P3 from **Y32A** digestion and **Y32A-Fna** was eluted as a single peak, showing that **Y32A** also had the same disulfide bond pairings as that of SPX (data not shown). Taken together, these results revealed that all the analogs including the four analogs (**R7A**, **Y10A**, **K30A**, and **Y32A**) had the same disulfide bond pairings as native SPX.

Inhibition of Kv1.3 potassium channels. We measured the blocking activity of native SPX and its synthetic peptide analogs on Kv1.3 channels at a concentration of 500 nM and also determined the IC₅₀ values of the analogs for Kv1.3 channels (Figure 5). Compared to native SPX (IC₅₀ = 63 nM), most alanine analogs showed similar or decreased blocking activity. Analogs **S4A**, **G5A**, **S6A**, **D8A**, **Q16A**, **T17A**, **P20A**, **N21A**, **I25A**, **S28A**, and **G33A** showed little or no (< 5%) difference in inhibitory activity at a concentration of 500 nM.

Notably, Ala substitutions of Lys²³, Asn²⁶, and Lys³⁰ resulted in the most significant decline of function, with **K23A** analog being completely inactive even at concentrations up to 500 μ M (Figure 5B) and **N26A** and **K30A** also losing inhibitory activity on Kv1.3 channels with IC₅₀ values of 12.7 ± 0.6 and 26.8 ± 0.7 μ M, respectively. In addition, Ala mutations of Arg⁷, Met¹⁴, Lys²⁷, and Tyr³² (analogs **R7A**, **M14A**, **K27A**, and **Y32A**, respectively) showed a shift of the IC₅₀ values to the micro molar range (1–3 μ M) indicating a significant decrease in Kv1.3 channel blocking activity.

CD spectra of SPX analogs. To analyze the secondary structure of peptides, we measured the CD spectra of each synthetic analog. Each spectrum showed that SPX and its analogs formed a typical scorpion CS α β toxin scaffold. The CD spectra of most analogs were nearly superimposable with that of native SPX, indicating that they have similar secondary structure to that of native SPX (Figure 6). The CD spectra of analogs **P12A**, **P20A**, and **G33A** slightly shifted from that of SPX.

DISCUSSION

In this study, we analyzed the role of each amino acid in the primary sequence of SPX using synthetic analogs in which each amino acid residue, except for Cys, was replaced by an Ala residue. We confirmed that all the purified analogs had the same disulfide bond pairings and secondary structures as that of native SPX. Ala substitutions of Lys²³, Asn²⁶, and Lys³⁰ in SPX resulted in significant loss of activity, identifying these residues as the most critical for inhibition of Kv1.3 channels (Figure 5). These three analogs (**K23A**, **N26A**, **K30A**) showed no change in their respective CD spectra compared with native SPX (Figure 6B), indicating that the reduced activity of these analogs is unlikely to be resulting from changes in secondary structure but rather to the loss of the side chains of Lys²³, Asn²⁶, and Lys³⁰. In particular, the substitution of Lys²³ resulted in complete loss of inhibition, suggesting that Lys²³ at the surface of the β -sheet is a key residue that determines binding to Kv1.3 (Figure 7). Interestingly, Lys²³ together with Tyr³² in SPX constitute the core “functional dyad” residues,

1 which are deemed to be critical for interaction with Kv channels and which are conserved
2 across many α -KTx6 subfamily members (see below).

3 We also noted that the CD spectra of analogs **P12A**, **P20A**, and **G33A** shifted slightly from
4 that of native SPX (see Figure 6) which could be accounted for by the fact that glycine and
5 proline are characteristic amino acids that determine the conformation of peptides.
6 Interestingly, **P20A** and **G33A** still retained their activities on Kv1.3 channels, whereas **P12A**
7 showed a decreased activity by ~7-fold compared to native SPX (Figure 5A), suggesting that
8 the conformational change induced by replacing proline with an alanine at position 12
9 resulted in the reduced activity.

10 SPX shares high sequence identity (82%) with MTX, from the scorpion *Scorpio maurus*
11 *palmatus*, which also belongs to the *Scorpionidae* family (Table 1). Brownian dynamics
12 simulations suggest that the critical triplet contacts in the structure of the MTX–Kv1 family
13 channel complex are Lys²³–His⁴⁰⁴ (Kv1 C chain), Lys²⁷–Asp³⁸⁶ (Kv1 B chain), and Lys³⁰–
14 Asp⁴⁰² (Kv1 D chain).²⁶ These three Lys residues in MTX are also conserved in SPX (Table
15 2). Lys²³ in MTX is predicted to protrude into the Kv1.2 pore,²⁶ and thus the key residue
16 Lys²³ in SPX may play a role similar to that of Lys²³ in MTX. Our experimental evidence
17 with SPX supports this by identifying the side chain of Lys²³ and Lys³⁰ as critical for Kv1.3
18 channel inhibition, while Lys²⁷ was one of the second most important residues. Thus, SPX
19 may be docked into the binding site of the Kv1.3 channel by primarily utilizing these three
20 Lys residues.

21 Many peptide toxins acting on voltage-gated Kv1 channels contain a “functional dyad” as a
22 key molecular determinant for their binding to Kv1 channels.^{21, 27, 28} A functional dyad
23 typically consists of a Lys residue and a hydrophobic residue, generally Tyr, Phe, or Leu,
24 separated by a distance of 6–7 Å. For example, Lys²³ / Tyr³² in MTX, Lys²³ / Tyr³² in Pi1, and
25 Lys²⁷ / Tyr³⁶ in charybdotoxin constitute the functional dyad in the respective toxins.^{21, 29, 30} It
26 is postulated that the side chain of Lys of the functional dyad protrudes deeply into the
27 selectivity filter, blocking the passage of potassium ions through Kv channels, while the
28 hydrophobic interaction of the other dyad residue with a cluster of ion channel aromatic

residues mostly accounts for the high-affinity binding of the toxin to the channel vestibule.²⁹
³¹ SPX also contains these amino acid residues (Lys²³ and Tyr³²). Whereas the substitution of
Lys²³ resulted in the complete loss of inhibition activity against Kv1.3 channel, that of Tyr³²
retained approximately 40% inhibition activity (Figure 5). This result shows that the
functional dyad of SPX is important but not essential for high affinity interaction with Kv1.3
channels.

A scorpion toxin Pi4 isolated from *Pandinus imperator* (*Scorpionidae*) shares high
sequence identity (85%) with SPX, and is a potent inhibitor of Kv1.2 channels (IC₅₀ = 8 pM)
(Table 2).⁹ The docking model of Pi4 on the Kv1.2 channel suggests that Arg¹⁰, Arg¹⁹, Lys³⁰
and Lys³³, which compose a ring of basic residues on the toxin, interact with the channel.⁹
SPX also has basic residues Arg⁷, Lys²⁷, and Lys³⁰ which correspond to Arg¹⁰, Lys³⁰, and
Lys³³ in Pi4, respectively. However, Arg¹⁹ in Pi4 corresponds to Gln¹⁶ in SPX. This difference
at position 16 reduces the positive electrostatic potential in this ring region of SPX and thus
might explain the lower activity of SPX on Kv1.2 channels compared to Pi4 (Table 1). We
also confirmed that the inhibitory activity of the analog **R7K**, where Arg⁷ was replaced with a
less basic Lys residue, was 13-fold weaker than native SPX in inhibiting Kv1.3 channels (data
not shown). These results suggest that the positive electrostatic potential on the surface of
SPX is required for a high affinity interaction with Kv1.3 channels.

Interestingly, the substitution of a non-basic amino acid Asn²⁶ with Ala in SPX resulted in
almost complete loss of activity (Figure 5). It has been reported that two amino acids (Lys and
Asn) at the specific positions, which correspond to Lys²³ and Asn²⁶ in SPX, are conserved as
part of a so-called *scorpion toxin signature* found in all α -KTx members.³² These Lys and
Asn residues are predicted to interact with Tyr³⁷⁵ and Asp³⁶¹ in Kv1.1 channels,
respectively.³² Therefore, Asn²⁶ in SPX may also play a similar role in the interaction with
Kv1.3 channels and the mutation to Ala most likely results in a less stable interaction of SPX
with the channel, as shown previously for other scorpion toxins.^{33, 34}

In the present study, we have experimentally identified the functionally important residues
in SPX for inhibition of Kv1.3 channels. As expected, Lys²³, Asn²⁶, and Lys³⁰ that are

conserved in the α -KTx6 subfamily were essential for high affinity interaction with Kv1.3 channels. On the other hand, substitutions of Arg⁷, Met¹⁴, Lys²⁷, and Tyr³² that are located at less conserved positions also largely reduced inhibitory activity, indicating that the amino acid residues at these positions may produce diverse inhibition activity for subtypes of Kv channels among α -KTx6 toxins. Comparison of the roles of amino acid residues among different members of the α -KTx6 subfamily may be able to provide valuable insights for the design of new drugs targeting Kv1.3 potassium channels with high potency.

ACKNOWLEDGEMENTS

We would like to thank Prof. Yasuyuki Shimohigashi and Prof. Yoshiki Katayama (Kyushu University) for the measurements of MALDI-TOF-MS and CD spectra. Thanks are also due to the students of Fukuoka Women's University involved in this project for their technical assistants in peptide synthesis. JT was supported by the following grants: G.0433.12 & G0E3414N (F.W.O. Vlaanderen) and IUAP 7/10 (Inter-University Attraction Poles Program, Belgian State, Belgian Science Policy).

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Table 1. Multiple sequence alignment of α -KTx6 family.

Unified name	Trivial name	Species	Amino acid sequence	Length (aa)	Sequence identity with SPX (%)	IC ₅₀ (nM)			Ref. No.
						Kv1.1	Kv1.2	Kv1.3	
α -KTx6.13	SPX	<i>Heterometrus Spinifer</i>	---IR C SGSRD C YSP C MKQTG C PN A K C INK S C K CY G - C --	34	100	NE	2.5	63	7
α -KTx6.4	Pi4	<i>Pandinus imperator</i>	IEA•••G•••••R••Q•R••••••••••T••••••S-	38	85	NE	0.008	NE	9
α -KTx6.2	MTX	<i>Scorpio maurus palmatus</i>	---VS•T••K••A••R••••••••••••••••••••	34	82	45	0.8	180	10, 11
α -KTx6.15	Hemitoxin	<i>Hemiscorpius lepturus</i>	---•K•TL•K•••••K•E••••R•••••RN•••••S-	35	74	13	16	2	20
α -KTx6.1	Pi1	<i>Pandinus imperator</i>	--LVK•R•TS••GR••QQ•••••S••••RM•••••--	35	65	NE	1.3	NE	21
α -KTx6.21	Urotoxin	<i>Urodacus yaschenkoi</i>	AGD•K•••T•Q•WG••K•••T•T•S•M•GK•••••VG	39	65	253	0.16	91	22
α -KTx6.3	HsTX1	<i>Heterometrus Spinifer</i>	---AS•RTPK•AD•R•E••••YG•M•RK•••NR•••--	34	53	7	NE	0.012	23, 24
α -KTx6.12	AnTx	<i>Anuroctonus phaiodactylus</i>	--QKE•T•PQH•TNF•R•N-K•THG•M•RK•••FN-•K-	35	39	NE	6.14	0.73	25

The list is an excerpt from α -KTx6 family which were reported for its activities on Kv channels.

Cysteine residues are highlighted in bold type, and dots indicate conserved residues. Numbers above indicate the positions of amino acids in SPX.

NE = No effect

Table 2. Mass spectrometry analyses of enzymatic fragments.

Peptide	Proteolytic fragment							
	Cys1–Cys5		Cys2–Cys6		Cys3–Cys7		Cys4–Cys8	
	Found [M + H] ⁺	Calcd [M + H]	Found [M + H] ⁺	Calcd [M + H]	Found [M + H] ⁺	Calcd [M + H]	Found [M + H] ⁺	Calcd [M + H]
SPX	984.83	984.15	735.40	734.83	848.53	848.06	795.52	794.89
I1A	984.29	984.15	734.79	734.83	848.27	848.06	795.08	794.89
R2A	1168.50	1168.39	735.09	734.83	848.14	848.06	794.99	794.89
S4A	968.54	968.15	735.25	734.83	848.45	848.06	795.15	794.89
G5A	ND	998.18	734.67	734.83	847.88	848.06	817.39	816.87
S6A	968.37	968.15	734.67	734.83	847.88	848.06	ND	794.89
R7A	-	-	-	-	848.25	848.06	795.24	794.89
D8A	984.07	984.15	690.85	690.82	847.84	848.06	795.00	794.89
Y10A	984.16	984.15	-	-	-	-	795.04	794.89
S11A	984.27	984.15	735.07	734.83	832.23	832.06	794.99	794.89
P12A	984.17	984.15	734.91	734.83	821.96	822.02	795.01	794.89
M14A	984.17	984.15	734.85	734.83	788.12	787.94	794.74	794.89
K15A	983.66	984.15	734.67	734.83	918.76	919.09	666.54	666.76
Q16A	984.23	984.15	734.90	734.83	847.84	848.06	738.04	737.84
T17A	983.48	984.15	734.48	734.83	847.91	848.06	764.61	764.86
G18A	983.86	984.15	734.74	734.83	847.78	848.06	808.74	808.92
P20A	984.00	984.15	734.73	734.83	847.63	848.06	968.08	968.10
N21A	983.90	984.15	734.53	734.83	847.61	848.06	950.81	951.12
K23A	1126.55	1126.31	735.15	734.83	848.25	848.06	795.17	794.89
I25A	942.33	942.07	734.89	734.83	847.86	848.06	795.08	794.89
N26A	941.20	941.13	735.02	734.83	848.13	848.06	794.99	794.89
K27A	856.10	855.98	805.91	805.91	847.80	848.06	795.08	794.89
S28A	984.27	984.15	719.07	718.83	848.13	848.06	794.92	794.89
K30A	984.32	984.15	-	-	-	-	795.13	794.89
Y32A	984.18	984.15	734.83	734.83	-	-	-	-
G33A	984.27	984.15	735.08	734.83	848.22	848.06	808.95	808.92

ND = Not Detected.

A hyphen indicates the fragments with two disulfide bonds, because that enzymatic cleavage sites were replaced with Ala.

The observed and theoretical value of the masses are indicated in the Found [M + H]⁺ and Calcd (*calculated*) [M + H] column, respectively.

Figure Legends

Figure 1. Amino acid sequence and disulfide bridges of SPX. Cysteine residues are indicated by the position number.

Figure 2. Representative example of RP-HPLC profile of a synthetic analog of SPX. The HPLC profiles of crude linear, crude oxidized, and purified oxidized of **I1A** are shown. Absorbance was measured at 230 nm. The theoretical and observed values of the masses are indicated as Calcd [M + H] and Found [M + H]⁺, respectively.

Figure 3. RP-HPLC profiles of enzymatic digestion of **I1A** (A) and **K30A** (B). Trypsin and chymotrypsin digestion sites are represented by closed and open triangle, respectively. Bold lettering indicates the substituted amino acid. An asterisk indicates fragments that were not digested by trypsin and chymotrypsin.

Figure 4. Synthetic scheme of reference peptides and determination of the disulfide bond pairing of **K30A**. (A) Bold lettering indicates the substituted amino acid. The protecting groups of side chains other than Cys are omitted for clarity. **K30A-Fna** had the same disulfide bond as SPX, whereas **K30A-Fnon** had a non-native disulfide bond. (B) The mixture of P3 from **K30A** digest and **K30A-Fna** was eluted as a single peak, showing that **K30A** had the same disulfide bond pairings as native SPX.

Figure 5. Inhibition of Kv1.3 channels by SPX and its analogs. (A) The bar indicates the percentage of inhibition at 500 nM concentration of toxin. Values are given as mean ± SE. Where determined, IC₅₀ values are shown at the right of the graph. ND is “not determined”. (B) Concentration-response curves for the inhibition of Kv1.3 by SPX and its analogs. Data for SPX and its seven analogs which lost or showed significantly reduced inhibitory activity against Kv1.3 channels are shown. The mutated positions in the amino acid sequence of SPX are also shown by the corresponding symbols as in the dose-inhibition curves for each analog of SPX. Amino acid residues, the mutation of which resulted in complete or near-complete loss of activity are depicted in red lettering, whereas mutated residues that resulted in significant loss of activity are shown in blue.

Figure 6. CD spectra of SPX and the analogs in H₂O solution. CD spectra of the analogs, (A) where a Pro or Gly was mutated to Ala; and, (B) which resulted in complete or near complete loss of activity are shown. Peptide concentration was 0.05 μmol/mL in all experiments.

Figure 7. The predicted structure of SPX. HsTX1 from *Heterometrus spinifer*¹⁷ (PDB accession number 1QUZ) was used as a template for homology modeling. The side chains of the five key residues located on the β -sheet are illustrated.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7