Chimia 55 (2001) 1058–1062 © Schweizerische Chemische Gesellschaft ISSN 0009–4293

Neurotoxic Peptides in the Multicomponent Venom of the Spider *Cupiennius Salei* Part I. Primary Structure of Neurotoxic Peptides in Relation to their Biological Function

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Abstract: Beside other components, spider venom contains neurotoxically acting peptides which predominantly target a variety of different ion channels within the spider's prey. From the multicomponent venom of *Cupiennius salei* 13 toxic peptides (CSTX-1 – CSTX-13) were purified by a combination of gel filtration, cation-exchange chromatography, and reverse-phase HPLC. The amino acid sequence of two important neurotoxic peptides, CSTX-1 (74 residues, 8352.6 Da) and CSTX-9 (68 residues, 7530.9 Da), as well as CSTX-2a (61 residues, 6865.7 Da), a truncated form of CSTX-1, were determined by Edman degradation. Sequence comparison of CSTX-1 with CSTX-9 revealed an identity of 53% as well as identical positions of all eight cysteine residues. CSTX-1 and CSTX-9 share the same disulphide bridge pattern linking Cys₁–Cys₄, Cys₂–Cys₅, Cys₃–Cys₈ and Cys₆–Cys₇. This disulphide bridge pattern is also found in other spider toxins such as agatoxins and curtatoxins. CSTX-1 and CSTX-9 belong to the family of the ion channel toxins containing the inhibitor cystine knot structural motif with the consensus sequence: **CX₃₋₇CX₃₋₁₀CX₀₋₇CX₁₋₈CX₄₋₂₀C.** CSTX-2a and CSTX-9, lacking the lysine-rich C-terminal tail of CSTX-1, exhibit a much lower toxicity for *Drosophila melanogaster* than CSTX-1, providing good evidence for the direct involvement of this lysine-rich C-terminal tail in the toxicity of CSTX-1.

Keywords: Amino acid sequence · *Cupiennius salei* · Inhibitor cystine knot structural motif · Pattern of the disulphide bridges · Spider toxins CSTX-1 - CSTX-13

1. Introduction

1.1. The Spider Cupiennius salei

The prey of spiders consists primarily of insects, however other arthropods and

occasionally even small vertebrates are also consumed. Most spiders inject venom into the body of their victims to paralyze them. This is achieved by biochemically active enzymes, proteins, peptides, and low molecular weight substances contained in the spider venom [1], of which some may act synergistically [2]. An important peptide fraction in spider venom is made up of neurotoxically acting peptides, which target different ion channels in the neuronal system of the spider's prey. Due to their extreme specificity, neurotoxins have become important tools in neurochemistry for the identification and characterization of diverse ion channels in both vertebrates and invertebrates.

Cupiennius salei Keyserling (Ctenidae) is a neotropical hunting spider living in forest habitats of Central America (Fig. 1). This large, nocturnal spider has a preference for large leaves of tall monocotyledonous plants such as banana plants (Musaceae), which simplifies the recognition of their prey via vibrations. In the last decades C. salei has become the most important and best studied laboratory spider. A recent review by Barth [3] is an excellent compilation of the available knowledge on the genus Cupiennius.

Originally, the investigations in the Nentwig group dealt with the toxic effects of different spider venoms [4] and the influence of the venom of C. salei on

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Fig. 1. The spider *Cupiennius salei* Keyserling (Ctenidae). The length of the white bar is 1 cm.

a variety of arthropod species [5]. Subsequently, we have isolated and characterized the most common components in the venom of *C. salei* [6–8]. However, many compounds are still under investigation; it is particularly their biological function that still needs to be elucidated.

1.2. The Significance of Edman Degradation

N-terminal amino acid sequence analysis by Edman degradation has been the work-horse for the sequence determination of peptides and proteins for many years, especially since the introduction of an automatic sequenator in 1967 by Per Edman himself. With the advent of DNA sequence analysis techniques in the midseventies, large-scale sequence projects gradually shifted from protein to DNA level. Sequence analysis of peptides by electrospray tandem mass spectrometry and by MALDI-TOF mass spectrometry in the early nineties introduced new options for protein identification and characterization. Nevertheless, Edman degradation still has merits in many areas, for instance in the field of the de novo sequence determination of unknown peptides and smaller proteins in species where a cDNA library is not available and/or the whole or parts of the genome are unknown.

The sequence determination of two important neurotoxic peptides, CSTX-1 and CSTX-9, in the venom of *C. salei* represents a good example of a fast and efficient sequence project by Edman degradation with state of the art instrumentation. In addition, their biological functioning in relation to their truncated forms CSTX-2a/CSTX-2b and CSTX-7 is discussed.

2. Results and Discussion

2.1. Isolation

A female adult spider contains about 10 µl of colorless, clear venom (pH 6.1), with an average protein content of 150 µg/µl. The multicomponent venom was purified by a combination of gel filtration, cation exchange chromatography and reverse-phase HPLC [6]. Gel filtration on a Superdex HR 75 column (Amersham Pharmacia, Sweden) yielded six main fractions. Fraction 2, containing peptides in the mass range of 4-14 kDa, exhibited the highest toxicity and was therefore further purified by cation exchange chromatography on a MonoS HR 10/10 column (Amersham Pharmacia, Sweden) yielding seven fractions, which were further separated by reverse-phase HPLC on a Nucleosil 300-5 C4 column (Macherey & Nagel, Germany). All 13 separated peptides (Fig. 2) revealed single bands on SDS-PAGE and exhibited toxicity to insects in bioassays, and were therefore termed *C. salei* toxins CSTX-1 – CSTX-13 [6].

2.2. Edman Degradation

Two important neurotoxic peptides [6-8], CSTX-1 (mass: 8351.90 ± 0.50 Da) and CSTX-9 (mass: 7530.25 ± 0.34 Da), as well as CSTX-2a (6864.88 ± 0.06 Da), a truncated form of CSTX-1, were subjected to sequence determination by Edman degradation (either in a Procise cLC 492 protein sequencer or in a pulsedliquid-phase sequencer 477A). The reduced and alkylated samples were sequenced up to Ile₅₃ (CSTX-1), Ala₄₇ (CSTX-9) and Phe₂₂ (CSTX-2a), respectively. In order to secure the rest of the sequences, reduced and alkylated CSTX-1 was cleaved with endoproteinase Asp-N and chymotrypsin, CSTX-9 with endoproteinase Asp-N and trypsin and CSTX-2a with endoproteinase Glu-C, respectively. In each case the peptides generated were separated by reverse-phase HPLC and identified by amino acid analysis and electrospray ionization mass spectrometry. The complete sequences of CSTX-1 and CSTX-9 with the corresponding overlaps are given in Figs. 3a and 3b, and

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Fig. 2. Isolation of the toxic peptides CSTX-1 – CSTX-13 from the spider *Cupiennius salei*. Fraction 2 from the gel filtration purification step was separated into fractions 1–7 by cation exchange chromatography and then further purified by reverse-phase HPLC on a Nucleosil 300-5 C4 column (4.6 x 250 mm; Macherey & Nagel, Germany). 13 peptides (CSTX-1 – CSTX-13) were isolated and final purification was achieved by an additional purification using reverse-phase HPLC.

the Glu-C peptides of CSTX-2a were aligned by comparison with CSTX-1 (Fig. 3c). The rather unique C-terminal sequence of CSTX-1, containing seven Lys residues within the last ten C-terminal amino acids, was verified with an alternative cleavage of native CSTX-1 with carboxypeptidase P, and subsequently the truncated forms of CSTX-1 were identified by electrospray ionization mass spectrometry. Thus, the C-terminal sequence was confirmed over a rather long stretch of 16 residues, *i.e.* from Lys₇₄ (C-terminus) to Ile₅₉ (Fig. 3a).

Sequence comparison of CSTX-9 with CSTX-1 revealed an identity of 53% as well as identical positions of all eight Cys residues present in the N-terminal portion of the molecule (Fig. 4). CSTX-2a represents a truncated form of CSTX-1 terminating at Arg_{61} , thus CSTX-2a lacks the lysine-rich C-terminal portion of CSTX-1. CSTX-2b (Ser₁-Phe₆₀) is a truncated form of CSTX-2a lacking Arg_{61} . CSTX-7 (mass: 7383.04 ± 0.18 Da) was identified by N-terminal sequence analysis (up to Asn₁₉), by amino acid composition and by mass spectrometry as a truncated form of CSTX-9 lacking the C-terminal Phe₆₈ residue. In addition, most noteworthy is the fact that CSTX-9 exhibiting a sequence identity of 53% with CSTX-1, also lacks the lysine-rich C-terminal tail of CSTX-1.

2.3. Disulphide Bridge Pattern and Inhibitor Cystine Knot Structural Motif

CSTX-1 (CSTX-2a/CSTX-2b) and CSTX-9 (CSTX-7) share the same disulphide bridge pattern linking Cys_1 - Cys_4 , Cys_2 - Cys_5 , Cys_3 - Cys_8 , Cys_6 - Cys_7 (the determination of the disulphide bridge pattern is given in the next article). This disulphide bridge pattern is also found in other spider toxins, e.g. ω -agatoxins (ω -AGA-IVA and ω -AGA-IVB) and μ -agatoxins (μ -AGA-I and μ -AGA-VI) from Agelenopsis aperta [9][10], SNX-325 from Segestria florentina [11] and curtatoxins (CT-I, CT-II and CT-III) from Hololena curta [12] (Fig. 4). Although the sequence similarity between CSTX-9/CSTX-1 and other spider toxins with four disulphide bridges is very low (Fig. 4), the disulphide bridge pattern is obviously identical. Many other spider toxins, such as agelenin [13] and huwentoxin [14], have only three disulphide bridges arranged in a 1-4, 2-5 and 3-6 pattern, according to the well-known consensus sequence of the inhibitor cystine knot structural motif:

 $CX_{3-7}CX_{3-8}CX_{0-7}CX_{1-4}CX_{4-13}C$ (X can be any amino acid, including Cys). The additional fourth disulphide bridge (C₆-C₇), present in CSTX-1/CSTX-9, can be thought of as an insertion between

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, 	Lys-Asp-Asp-Lys-Asn-Cys-Ile-Pro-Lys-His-	
CSTX-9	r/a His-Glu-Cys-Thr-Asn-Asp-Lys-Lys-Asn-Cys-	
CSTX-9	25 Cys-Lys-Lys-Gly-Leu-Thr-Lys-Met-Lys-Cys-	
CSTX-9	³⁵ Lys-Cys-Phe-Thr-Val-Ala-Asp-Ala-Lys-Gly-	
CSTX-9		
Азр-И	N4	
	45 Ala-Thr-Ser-Glu-Arg-Cys-Ala-Cys-Asp-Ser-	
CSTX-9		
Asp-N	55 Ser-Leu-Leu-Gln-Lys-Phe-Gly-Phe-Thr-Gly-	
Asp-N		
	65 69	
	65 60 Leu-His-Ile-Ile-Lys-Gly-Leu-Phe	
Asp-N	65 60 Leu-His-Ile-Ile-Lys-Gly-Leu-Phe	

5 C) Ser-Cys-Ile-Pro-Lys-His-Glu-Glu-Cys-Thr-CSTX-2a r/a Glu-C E2 -15 Asn-Asp-Lys-His-Asn-Cys-Cys-Arg-Lys-Gly-CSTX-2a Glu-C Leu-Phe-Lys-Leu-Lys-Cys-Gln-Cys-Ser-Thr-CSTX-2a Glu-C 35 Phe-Asp-Asp-Glu-Ser-Gly-Gln-Pro-Thr-Glu-Glu-C - E1a Arg-Cys-Ala-Cys-Gly-Arg-Pro-Met-Gly-His-Glu-C Gln-Ala-Ile-Glu-Thr-Gly-Leu-Asn-Ile-Phe-Glu-C ---- E1b ------Arg Glu-C _____

Fig. 3a-c. Determination of the amino acid sequences of CSTX-1, CSTX-9 and CSTX-2a by Edman degradation. The sequences were determined by Edman degradation using reduced and alkylated CSTX-1, CSTX-9 and CSTX-2a, as well as peptides generated from cleavages with endoproteinase Asp-N, endoproteinase Glu-C, trypsin and chymotrypsin, respectively. In addition, the unique C-terminal sequence of CSTX-1 was verified using a digestion with carboxypeptidase P (CPP), with a subsequent identification of the truncated fragments by electrospray ionization mass spectrometry.

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	10	20
SNX-325	GSCIESG-KSCTH	ISRSMKNGLUUPKSK
μ -AGA-I	ECVPEN-GHCRD	WYDE CCEGFY -
ω-AGA-IVA	KKKCIAKDYGRCKW	IGGTPCCRGRG-
CT-I	SCVGEY-GRCRS	SAYED C C D G Y Y -
CSTX-1	SCIPKH-EECTN	IDKHNCCRKGLF
CSTX-9	DKNCIPKH-HECTN	IDKKNCCKKGLT
SNX-325 μ-AGA I ω-AGA IVA CT I CSTX-1 CSTX-9	30 -40 - C N C R Q I Q H R H D Y L - C S C R Q P P K	50 G K R K Y S C R C S C I C R N N N C E C K P R L I M C L C R N N N P T E R C A C G R P M G H T S E R C A C D S S L L Q
ω-AGA IVA CSTX-1 CSTX-9	60 68 SLGLA IETGLNIFRGLFKG GFTGLHIIKGLF	ЗКККИККТК

Fig. 4. Comparison of a selection of spider toxins with the same disulphide bridge pattern containing the inhibitor cystine knot structural motif. Amino acids identical with the toxins CSTX-1 and CSTX-9 are shaded in gray. The Cys residues of the pattern are shaded in gray and marked within boxes and are linked Cys_1-Cys_4 , Cys_2-Cys_5 , Cys_3-Cys_8 and Cys_6-Cys_7 . CSTX-1/CSTX-9: *Cupiennius salei*; agatoxins μ -AGA-I/ ω -AGA-IVA: *Agelenopsis aperta* [9][10]; SNX-325: *Segestria florentina* [11]; curtatoxin CT-I: *Hololena curta* [12].

bridge 3–6 of the inhibitor cystine knot structural motif, as has already been demonstrated for ω -AGA-IVA/ ω -AGA-IVB and μ -AGA-I/ μ -AGA-IV [15][16]. Taking into account the extended length of this insertion with 17 residues for CSTX-1/ CSTX-9 and even 20 residues for SNX-325, and in order to be able to satisfy all known examples, the inhibitor cystine knot structural motif has to be modified into the following consensus sequence:

CX3-7CX3-10CX0-7CX1-8CX4-20C

2.4. The Biological Function in Relation to the Structure

CSTX-9 lacks the lysine-rich, ten residues long C-terminal tail of CSTX-1 (Fig. 4). Testing CSTX-9 in the *Drosophila melanogaster* bioassay, the LD₅₀ was 3.12 pmol/mg fly, thus exhibiting a ninefold lower toxicity than CSTX-1. This observation is in accordance with previous data of two truncated forms of CSTX-1, CSTX-2a (Ser₁-Arg₆₁) and CSTX-2b (Ser₁-Phe₆₀), both also lacking the lysine-rich C-terminal tail of CSTX-1. They exhibit a sevenfold and 190-fold reduced activity to *Drosophila melanogaster*, respectively, a clear indication of the direct involvement of the lysinerich C-terminal tail of CSTX-1 in the toxicity [7].

Acknowledgements

We thank Michèle Stark for critically reading the manuscript. This research was supported by the Swiss National Science Foundation.

Received October 17, 2001

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