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Primary structure of scorpion anti-insect toxins isolated from the venom of *Leiurus quinquestriatus quinquestriatus*

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The amino acid sequences of insect-selective scorpion toxins, purified from the venom of *Leiurus quinquestriatus quinquestriatus*, have been determined by automatic phenyl isothiocyanate degradation of the *S*-carboxymethylated proteins and derived proteolytic peptides. The excitatory toxin Lqq IT_1 and Lqq IT_1' (70 residues) show the shift of one half-cystine from an external position, which is characteristic of anti-mammal toxins, to an internal sequence position. Lqq IT_2 (61 residues) displays the half-cystine residue in position 12, common to the sequence of all known anti-mammal toxins; it induces flaccid paralysis on insects but is non-toxic for the mouse. Lqq IT_2 structurally defines a new type of anti-insect toxins from scorpion venoms. CD spectra and immunological data are in agreement with this finding.

Scorpion venom; Anti-insect toxin; Primary structure

1. INTRODUCTION

Scorpion venoms contain numerous toxins active on the potential-dependent sodium channels [1]. The range of animal species sensitive to a particular toxin is generally limited to a phylogenetic class. Thus, toxins specifically active on mammals [2,3], insects of crustaceans [4] have already been described. One structural basis of the selectivity of anti-insect toxins has been related to a difference in the position of one of the 4 disulfide bridges which are present in anti-mammal [5] and anti-insect toxins [6]. The species selectivity is not absolute since it was found that an anti-mammal toxin, i.e. Ts VII from *Tityus serrulatus* [7], was active on insects [8] and able to compete with AaH IT_1 for binding to insect synaptosomal fractions [9]. Lqq IT_1 and Lqq IT_2, from *Leiurus quinquestriatus quinquestriatus*, are two anti-insect toxins which give rise to different symptomatologies in vivo: Lqq IT_1 induces a fast excitatory contraction paralysis on fly larvae whereas Lqq IT_2 induces a slow, depressant, flaccid paralysis [10]. However, they both compete with AaH IT for binding to a nerve synaptosomal fraction of the cockroach *Periplaneta americana* [10]. We found it necessary to structurally define these two toxins by establishing their amino acid sequences and assessing their antigenic and circular dichroism properties.

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Abbreviations: AaH IT, AaH IT_1, AaH IT_2, *Androctonus australis* Hector anti-insect toxins; Lqq IT_1, Lqq IT'_1, Lqq IT_2, *Leiurus quinquestriatus quinquestriatus* anti-insect toxins; Ts VII, *Tityus serrulatus* toxin VII; RCM-toxin, reduced and *S*-carboxymethylated toxin; CpA, CpY, carboxypeptidases A and Y; V- or C- peptides, peptides obtained by action of *Staphyloccus aureus* V8 protease or chymotrypsin, respectively; RIA, radioimmunoassay; CD, circular dichroism; PTH, phenylthiohydantoin

2. MATERIALS AND METHODS

2.1. *Toxins purification*

Lqq IT_1 and Lqq IT_2 were isolated from 5 g of *Leiurus q.q.* venom (Sudan) according to Zlotkin et al. [10]. However, for Lqq IT_2, an additional HPLC purification step was carried out using a Beckman RP-8 column (10 × 250 mm). Elution was performed with a 70 min gradient from 15% to 40% acetonitrile in ammonium formate (0.15 M, pH 2.7), at a flow rate of 4.9 ml/min. Absorbance detection was performed at 280 nm.

2.2. *Amino acid sequence determination*

Reduction and *S*-carboxymethylation by iodo 2-[^{14}C]acetic acid were performed on 100 nmol of each toxin [7,11]. Forty nmol of both RCM-toxins were digested by the *Staphyloccus aureus* V8 protease [12]. In the case of Lqq IT_2, peptides were also generated by the action of chymotrypsin (2% w/w) on 15 nmol RCM-toxin (0.1 M HCO_3NH_4, pH 7.8, 2 h, 37°C). All peptides were purified by HPLC. C-terminal residues of Lqq IT_1 were identified following the action of 20% (w/w) CpA on 2.5 nmol RCM-toxin (0.2 M *N*-ethyl-morpholine, pH 8.0, 37°C). The C-terminal sequence of Lqq IT_2 was obtained by action of 15% (w/w) CpY on 2.5 nmol RCM-toxin (0.05 M sodium acetate, pH 5.5, 20°C); aliquots were removed at time intervals for amino acid analyses (Beckman 6300 analyzer). Automated Edman degradations of RCM-toxins and peptides were performed on a Beckman 890 M Sequencer using the 0.1 M Quadrol programme. PTH-amino acids were identified according to [13]. *S*-[^{14}C]carboxymethyl-cysteine PTHs were also identified by radioactivity measurements.

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2.3. *Biological assays*

RIA were carried out in solid-phase conditions according to El Ayeb et al. [14] using the various toxins and a purified IgG fraction from a rabbit anti-AaH IT serum. Toxicity for mice was tested by injecting increasing amounts of Lqq IT_2, using an intracerebroventricular route. Toxicity for insects was measured on blowfly larvae as described in [10].

2.4 *Circular dichroism*

Circular dichroism spectra of solutions of proteins (0.2 mg/ml) were recorded on a Jobin-Yvon Mark V Autodichrograph.

3. RESULTS AND DISCUSSION

When prepared according to [10], Lqq IT_2 was found, by HPLC, to be contaminated by 3 other fractions. The main peak (70% of the material) induced flaccid paralysis in 50% of injected blowfly larvae, at a dose of 140 ng/larva, and was used for further studies. The amino acid compositions of RCM-Lqq IT_1 and RCM-Lqq IT_2 are reported in tables 1 and 2, respectively. Lqq IT_1 is composed of 70 amino acids, while Lqq IT_2 is composed of 61 residues and not 72 as previously published [10]; moreover, it contains one proline residue and no valine.

The N-terminal sequence of Lqq IT_1 was established up to the Ile-56 residue by automatic degradation of 15 nmol RCM-toxin (fig.1A). The last 5 residues were identified using CpA. The gap between Ile-56 and Phe-66 was filled by the sequencing of peptide V_V (positions 56–67). However, during the degradation, aspartic and glutamic acid-PTHs (50% each) were found in position 33. The appearance of two PTHs at this position is consistent with the simultaneous sequencing of two isotoxins, which were not separated by ion-exchange chromatography or HPLC; it readily explains the amino acid composition of Lqq IT_1 (Asp and Glu: table 1). Fig.1A summarizes the results.

The first 32 residues of Lqq IT_2 were determined by automatic degradation of 2.5 nmol RCM-toxin (fig.1B). The last 4 residues were obtained by the action of CpY on RCM-toxin. The C- and V-peptides were purified by HPLC (table 2), and the amino sequences of some of these were established. The analyses of the sequences of peptides V_{IIIb}, V_X, C_{III}, and C_{XIV} made it possible to obtain the necessary overlaps and to deduce the complete amino acid sequence of Lqq IT_2.

In a solid-phase RIA (not illustrated), it was found that Lqq IT_1 is fully recognized by anti-AaH IT antibodies. In contrast, Lqq IT_2 does not display any significant immunoreactivity in this assay. Antigenic similarities between Lqq IT_1 and AaH IT were previously used in the affinity chromatography purification of anti-insect toxins from different venoms of *Buthidae* scorpions [15]. It was concluded that the excitatory anti-insect toxins formed a family of strongly homologous proteins. These observations, are supported by this work and the one of Loret et al. [16], who recently isolated two homologous excitatory toxins (AaH IT_1 and AaH IT_2) from AaH venom (fig.1C). The CD spectra of Lqq IT_1 and Lqq IT_2 are very dif-

Table 1

Amino acid compositions of RCM-Lqq IT_1 and V-peptides derived from it

	S. aureus V_8 peptides				
Amino acids	V_{III}	V_{VI}	V_{IX}	V_V	RCM Lqq IT_1
CM cysteine		1.8 (2)	4.3 (5)	0.9 (1)	8.1 (8)
Aspartic acid	2.0 (2)	2.0 (2)	3.6 (3)	2.9 (3)	9.6 (9–10)[a]
Threonine			1.0 (1)	0.9 (1)	1.8 (2)
Serine	1.6 (2)	0.9 (1)	2.0 (2)	0.8 (1)	5.8 (6)
Glutamic acid	1.1 (1)	0.9 (1)	1.3 (1)		3.5 (3–4)[b]
Proline	1.0 (1)				0.9 (1)
Glycine	1.9 (2)		2.2 (2)		4.2 (4)
Alanine	2.0 (2)		0.9 (1)	1.1 (1)	3.9 (4)
Valine	1.0 (1)		2.6 (3)	1.0 (1)	4.8 (5)
Isoleucine				2.0 (2)	1.7 (2)
Leucine		1.6 (2)	3.4 (4)		5.2 (6)
Tyrosine	0.9 (1)	1.6 (2)	2.4 (3)	0.8 (1)	4.9 (7)
Phenylalanine				1.0 (1)	1.0 (1)
Histidine			0.9 (1)		0.8 (1)
Lysine	2.8 (3)		4.0 (4)	2.2 (2)	8.4 (9)
Arginine				0.9 (1)	0.9 (1)
Sequence positions	1–15	16–25	26–55	56–70	1–70
Elution time (min)	5.6	12.3	22.0	12.0	Molecular mass 8090

Numbers in brackets correspond to residues determined by sequencing; [a] = (10) for Lqq IT_1; [b] = (4) for Lqq IT_1'. Peptides were purified by HPLC. (Column Aquapore RP-300-5 μm; 4.6 × 30 mm); linear gradient from 5% to 28% acetonitrile (0.1% TFA) for 10 min; then 28% to 40% for 16 min; flow rate 1 ml/min; 215 nm detection

Table 2

Amino acid compositions of RCM-Lqq IT_2 and of proteolytic peptides derived from it

	S. aureus V-peptides[a]					Chymotryptic peptides[b]			RCM Lqq IT_2[c]
	V_{III}[d]			V_{VII}	V_x	C_{III}	C_{VII}	CT_{XIV}	
Amino acids	V_{IIIa}	V_{IIIb}	V_{IIIc}						
CM cysteine				2.1 (2)	2.8 (4)	1.0 (1)	1.4 (2)	1.1 (1)	7.6 (8)
Aspartic acid	1.0 (1)		2.3 (2)	1.3 (1)		1.0 (1)	1.7 (2)	1.1 (1)	6.0 (6)
Threonine		0.9 (1)			0.9 (1)	1.7 (2)		0.7 (1)	3.6 (4)
Serine		1.1 (1)		0.9 (1)	2.1 (2)	1.0 (1)	1.3 (1)	0.4 (1)	4.9 (5)
Glutamic acid	1.0 (1)	1.3 (1)		1.4 (1)	1.5 (1)	1.0 (1)	1.9 (2)	2.1 (2)	5.0 (5)
Proline	1.0 (1)							0.9 (1)	1.0 (1)
Glycine	1.2 (1)			2.0 (2)	4.1 (4)	1.3 (1)	2.5 (2)	1.3 (1)	9.7 (10)
Alanine					1.4 (1)				1.5 (1)
Isoleucine			0.9 (1)						0.8 (1)
Leucine	1.0 (1)			1.9 (2)	1.3 (1)			1.0 (1)	3.8 (4)
Tyrosine			0.8 (1)		1.7 (3)		1.0 (1)		3.5 (4)
Phenylalanine				0.9 (1)					1.0 (1)
Lysine		2.1 (2)		1.0 (1)	1.0 (1)	1.0 (1)	2.4 (2)	0.9 (1)	6.0 (6)
Tryptophane		n.d. (1)			n.d. (3)				n.d. (4)
Arginine			1.8 (2)						1.9 (2)
Sequence positions	46–50	51–56	1–8	9–19	25–45	64–61	17–28	44–53	1–61
Elution time (min)	4.2/31.4	4.2/33.0	4.2/33.5	20.0	26.2	10.7	15.4	33.6	Molecular mass 7083

Residue numbers in brackets were those found by sequencing. n.d. = not determined. [a] Peptides obtained on RP-18 LiChrospher (4 × 125 mm). Linear gradient from 20% to 70% acetonitrile (0.1% TFA) for 35 min (0.6 ml/min. [b] C-peptides eluted with 5% acetonitrile for 10 min, followed by a linear gradient from 5% to 60% in 55 min (0.6 ml/min. [c] The number of residues is the average of two 24-h and two 72-h hydrolyses in 6.0 N HCl at 110°. [d] Further chromatography was performed for V_{III} in 5% acetonitrile (0.1% TFA) for 10 min, followed by a linear gradient from 5% to 40% in 35 min; 215 nm detection

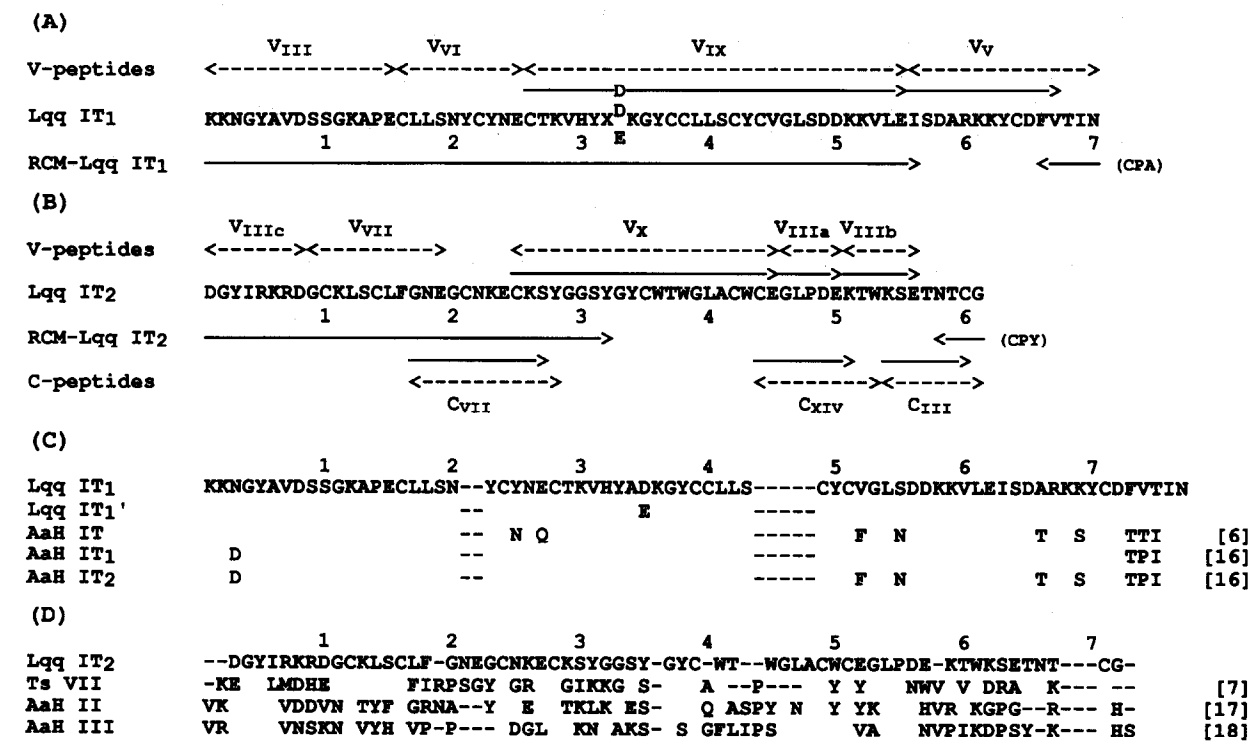

Fig.1. Primary structures of Lqq IT_1 (A) and Lqq IT_2 (B). Amino acid positions are counted in tens. Arrows in full line represent the residues identified by automatic sequencing (——→), and amino acids released by carboxypeptidases (←——); arrows in dotted line (<---->) represent the peptides obtained either by *Staphylococcus aureus* V8 protease (V-peptides) or by chymotrypsin (C-peptides). (C) Comparison of the amino acid sequence of Lqq IT_1 with Lqq IT_1' and with excitatory anti-insect toxins from *Androctonus australis* Hector. (D) Comparison of the amino acid sequence of Lqq IT_2 with AaH II, AaH III (α-type anti-mammal toxins), and with Ts VII (β-type toxin, active on mammals and insects). Lqq IT_1 (C) or Lqq IT_2 (D) sequences are taken as references. Only differing amino acids are indicated for other toxins. Deletions are introduced for alignment of sequences of panels (C) and (D).

ferent (not illustrated), suggesting that the two proteins probably differ in their secondary structures. The sequence results reported here may explain the differences in their antigenic and conformational properties. It appears from fig.1 that the amino acid sequence of Lqq IT_2 has less in common with the family of excitatory anti-insect toxins (fig.1C) than with anti-mammal toxins (fig.1D). In particular, the half-cystine present in position 12 of Lqq IT_2 is absent in AaH IT and Lqq IT_1 [5,6]. Homologies were alternatively found with AaH II, AaH III and Ts VII. Despite these similarities with anti-mammal toxins, Lqq IT_2 showed no toxic effect by intracerebroventricular injections in mice, up to the high dose of 20 μg/kg (above-mentioned anti-mammal toxins are lethal to mice at doses lower than 0.03 μg/kg). Binding experiments [8] showed that the fixation of ^{125}I-AaH IT_1 was inhibited by Lqq IT_1, AaH IT_1 and AaH IT_2, respectively and, more unexpectedly, by the depressant toxin Lqq IT_2 and by Ts VII. These results clearly indicate that all these toxins compete for fixation on the same insect nervous system receptors, despite major differences between their sequences.

Contrary to Ts VII, active on both mammals and insects, Lqq IT_2 is found here to be active only on insects. Considering the antigenic behaviour, CD spectra and amino acid sequence of Lqq IT_2, it is clear that depressant toxins constitute another group of anti-insect toxins with a typical chemical and antigenic structure. The occurrence of other toxins of the same type was recently confirmed, since Zlotkin et al. [19] described very homologous toxins, including Lqq IT_2, that were isolated from different species of *Buthidae* scorpions.

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