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Biochemical, pharmacological and genomic characterisation of Ts IV, an α-toxin from the venom of the South American scorpion *Tityus serrulatus*

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Abstract

The venom of the scorpion, *Tityus serrulatus*, was fractionated to investigate the chemical and pharmacological properties of its α-toxin content. Three α-toxins (Ts III, Ts IV and Ts V) were purified by conventional chromatography (gel filtration and ion-exchange chromatography), followed by immunoaffinity chromatography. Competition experiments using reference α- and β-toxins suggested that these α-toxins were contaminated with around 0.01% of β-toxin. The sequence of the first 30 amino acids of Ts IV was established. Using an oligonucleotide probe, a cDNA encoding its precursor was cloned from a venom gland cDNA library. The primary structure deduced from the cDNA nucleotide sequence provides possible explanations for the polymorphism of these three molecules.

Key words: α- and β-Scorpion toxin; cDNA cloning; *Tityus serrulatus*

1. Introduction

The study of the venom from the Brazilian scorpion, *Tityus serrulatus*, by numerous authors using different methods has led to the isolation of several toxins [1–5]. Two types of toxins (α- and β-toxin) have been characterized in this venom according to their specific binding to sites 3 and 4, respectively, on the voltage-gated sodium channel of rat brain synaptosomes [5,6] and their different electrophysiological activities (α-toxins inactivate the channel and β-toxins act at the activation of the channel) [7,8].

The most potent β-toxin is Ts VII, also called Ts γ [2,5–7,9,10]. We recently reported the cDNA nucleotide sequence encoding the Ts VII precursor [11].

The preparation containing one α-toxin, which was the first toxin purified from *T. serrulatus* venom (because of its high toxicity for mammals by subcutaneous injection), was previously termed Tityustoxin [1,6,8]. The primary structure of this molecule was not determined. The sequence of the first 55 amino acids of the α-toxin IV-5 (which is probably the same molecule as Tityustoxin [1,2], has been published [3]. Recent work assumed that Tityustoxin was highly heterogeneous and that its various reported biological activities were due to the presence of several different components [4]. As Tityustoxin has often been used in biological and electrophysiological studies, it would be valuable to define its binding characteristics and to completely elucidate its primary structure. We therefore investigated the chemical and pharmacological properties of this toxin, using genetic and others approaches.

2. Materials and methods

Tityus serrulatus venom (1 g) was obtained from scorpions collected near Santa Barbara MG, Brazil. Sephadex G50 was from Pharmacia and the CM-cellulose CM_{52} from Whatman Inc. The toxicity of the chromatographic fractions was assayed either by subcutaneous (s.c.) or intracerebroventricular injection (i.c.v.) into C57 Bl/6 mice [12]. Polyacrylamide gel electrophoresis was performed at pH 4 on a 20% homogeneous Phast-gel using a Phastsystem according to the manufacturer's instructions (Separation file 121, development file 200). Amino acid analysis was performed using a Beckman 6300 analyzer, and automatic sequencing of the toxin was performed in a Beckman 890 C microsequencer as described [12]. Reference α- and β-toxins (i.e. AaH II and Css II, respectively) and Ts VII were purified in the laboratory and radioiodinated according to published procedures [9,13]. Binding competition assays with ^{125}I-labeled α- or β-toxin on rat brain synaptosomes were performed as previously described [13]. Polyclonal sera against Ts VII were obtained in New Zealand rabbits and were characterized as described [14]. Rabbit anti-Ts VII IgG was purified on an Affipak pre-packed column (Pierce Chemical Co.) and immobilized on CNBr–Sepharose for purification of Ts IV by affinity chromatography. Radioimmunoassay (RIA) against [^{125}I]Ts VII (2×10^{-10} M) was performed with Ts IV submitted or otherwise to immunoaffinity chromatography using Ts VII antiserum (diluted 1/100) as described [14].

Standard recombinant DNA techniques were used [15]. Enzymes were from Biolab. *Escherichia coli* strains C600 hfl and JM 109 were

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used for phage production. The construction of the venom gland cDNA library has previously been described in detail [11]. The λgt10 library contained 2.5×10^6 independent phage clones. The oligonucleotide probe used to screen the library was synthesized using an Applied Biosystems Model 391 DNA synthesizer. The sequence of the degenerate probe was 5′ TG(CT)TGGAA(CT)TA(CT)GA(CT)AA(CT)GC3′ and corresponded to the nucleotide sequence encoding amino acids 16–22, as determined by Edman degradation. The probe was ^{32}P-end labelled using T4 polynucleotide kinase. 750,000 clones from the cDNA library were analyzed as previously described [11,15]. The cDNA inserts were excised from positive phage DNA with *Eco*RI and were subcloned into M13 mp18 for sequencing [11,15].

3. Results

The *T. serrulatus* venom was dialysed against distilled water to eliminate salts and small peptides of $M_W < 3{,}500$. Gel filtration through Sephadex G50 gave four toxic fractions (labelled S1 to S4 in Fig. 1A). Fraction S4, which was able to displace the radiolabelled α-toxin ([^{125}I]AaH II) from its binding site on rat brain synaptosomes, was further separated on CM-cellulose CM52 into four toxic fractions (Fig. 1B): each of the fractions were homogeneous as assessed by polyacrylamide gel electrophoresis and staining for total protein (data not shown). The toxins were numbered Ts III, Ts IV, Ts V and Ts VIII [5]. Their LD_{50} per 20 g mouse were: 2.5 μg, 0.4 μg, 0.2 μg and 7.2 μg, respectively, as determined by s.c. injection, and 20 ng, 24 ng, 15 ng and 11 ng, respectively, by i.c.v. injection. The amino acid composition of Ts IV, the most active fraction by s.c. injection, was as follows: 11 Asp, 2 Thr, 3 Ser, 2 Glu, 3 Pro, 4 Gly, 3 Ala, 8 Cys, 2 Val, 2 Ile, 3 Leu, 8/9 Tyr, 1 His, 9 Lys, 2 Trp (spectrophotometrically determined). Met, Phe and Arg were absent. The amino acid compo-

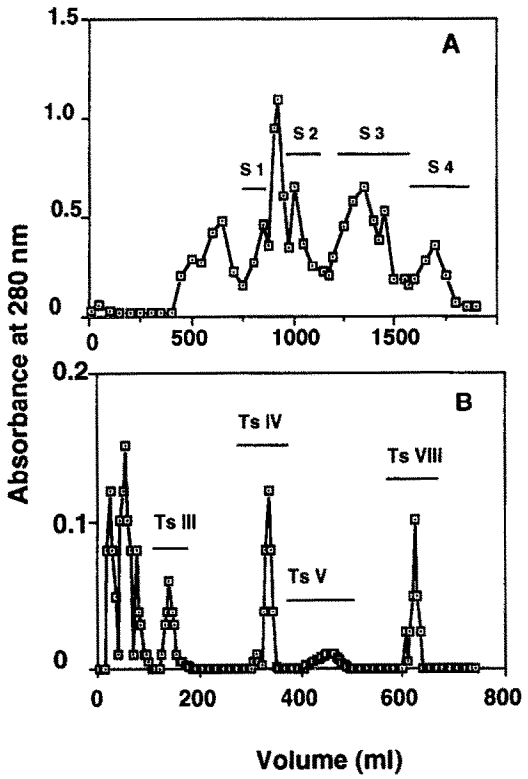

Fig. 1. Purification of Ts IV. (A) Gel-filtration through Sephadex G50 in 0.1 M ammonium acetate buffer, pH 8.5; four columns (2.5 × 100 cm) in series; flow rate, 18 ml/h; S1–S4, fractions toxic to mice by s.c. injection. (B) Chromatography on CM-cellulose CM52 of the fraction S4 obtained in A; column 1 × 40 ml; elution buffers, equilibrium step (300 ml), 0.02 M ammonium acetate, pH 7, followed by an ammonium acetate gradient from 0.02 M, pH 7 (100 ml) to 0.15 M, pH 7 (100 ml); a second short equilibrium step (50 ml) of 0.15 M was followed by a gradient from 0.15 M, pH 7 (100 ml) to 2 M, pH 7 (100 ml); flow rate, 20 ml/h.

A

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.........TTG GTC GTC GTC TGC TTA TTG ACC GCG GGC ACG
          L   V   V   V   C   L   L   T   A   G   T

GAG GGC AAG AAA GAC GGA TAC CCG GTG GAA TAC GAT AAC
 E   G   K   K   D   G   Y   P   V   E   Y   D   N
         +1                                  +10

TGC GCC TAC ATT TGC TGG AAC TAC GAC AAC GCT TAC TGC
 C   A   Y   I   C   W   N   Y   D   N   A   Y   C
                                +20

GAT AAG CTG TGC AAA GAC AAG AAA GCC GAT AGC GGA TAT
 D   K   L   C   K   D   K   K   A   D   S   G   Y
                    +30

TGT TAC TGG GTT CAC ATC CTG TGC TAC TGC TAC GGG CTT
 C   Y   W   V   H   I   L   C   Y   C   Y   G   L
    +40                                        +50

CCC GAT AGC GAA CCG ACC AAG ACC AAC GGA AAA TGC AAA
 P   D   S   E   P   T   K   T   N   G   K   C   K
                                    +60

TCC GGT AAG AAG TAA ACCAGCCTCCTATTGATCCCAGATCCGCCCT
 S   G   K   K   *

GGCGATAAATGTTTCTGAAAACCATTCCCGAAATAAAACTCATGCCTGCA

AAAAAAAAAAAAAAA
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B

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Ts III:        ...G   K   C-OH
                 +60

Ts IV:         ...G   K   C   K   S-NH2
                 +60

Ts V:          ...G   K   C   K   S   G   K-OH
                 +60                +65

Precursor:     ...G   K   C   K   S   G   K   K-OH
                 +60                +65
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Fig. 2. Nucleotide sequence of the cDNA encoding the Ts IV precursor. (A) The predicted protein sequence is given below the nucleotide sequence and is numbered starting from the N-terminal amino acid residue of the toxin; the signal peptide sequence is underlined; the stop codon is designated by an asterisk; a potential polyadenylation signal is underlined twice. (B) Putative C-terminal sequences of Ts III, Ts IV and Ts V deduced from the possible different processing of the same precursor.

sitions of Ts III and Ts V were quite similar: Ts III and Ts V only differed from Ts IV by 1 fewer Lys in Ts III and by an additional Lys and Gly in Ts V. Toxin Ts VIII has exactly the same amino acid composition as Ts VII, a β-toxin.

The N-terminal amino acid sequence of Ts IV was established up to the 30th residue (except positions 12, 16 and 24, which, by homology to other scorpion toxins, should be Cys) by automatic Edman degradation of 50 nmol of native toxin and was found to be identical to toxin IV-5 [2,3].

The *T. serrulatus* cDNA library was probed with a ^{32}P-labelled oligonucleotide designed from amino acids 16–22 of Ts IV, assuming that residue 16 was a Cys. Forty-two positive clones were selected and analyzed by restriction mapping. The twelve longest inserts (sized between 290 and 440 bp) were subjected to sequence analysis and found to encode the Ts IV precursor (Fig. 2A). They differed from each other only by the length of the 5′ sequence and 3′ poly(A) tail. Even in the longest insert analyzed, no in-frame Met initiation codon was found, probably due to incomplete elongation during the cDNA synthesis by reverse transcriptase. Thirty of the encoded amino acid residues match the N-terminal sequence of Ts IV and 55 that of toxin IV-5 [3]. Gly-Lys-Lys residues terminate the toxin precursor. Identically, the Ts VII precursor [11] contains a Gly-Lys-Lys sequence at the C-terminal, not present in the mature Ts VII. These three residues are presumably processed, the Lys residues being removed by a carboxypeptidase and the remaining Gly-extended peptide being converted into a des-Gly peptide amine by an α-amidating enzyme to give an α-amidated C-terminal end. Previous reports describe similar processing steps for the C-termini of other scorpion toxin precursors [12,16]. Thus, we suppose that the C-terminal residue (residue 64) of the mature Ts IV is a serine-amide. From the precursor sequence of Ts IV deduced from the cDNA, it is also tempting to propose that Ts III and Ts V are immature forms of Ts IV: digestion of the Ts IV C-terminal residues, i.e. Lys63 and Ser64, by exopeptidase would give Ts III; Ts V could exhibit an incompletely processed C-terminus and two additional residues: Gly65-Lys66 (Fig. 2B).

In receptor-binding experiments (Fig. 3A and B), Ts III, Ts IV and Ts V all inhibited the binding of the α-toxin, [^{125}I]AaH II ($K_{0.5}$ of 10^{-5} M, 3×10^{-7} M and 10^{-7} M, respectively) and the β-toxin [^{125}I]Css II ($K_{0.5}$ of 10^{-5} M, 10^{-7} M and 3×10^{-5} M, respectively). Thus, each toxin seems to bind both sites 3 and 4 on the sodium channel. Ts IV also inhibited the binding ($K_{0.5}$ ratio = 1/10,000) of the β-toxin [^{125}I]Ts VII to Ts VII antibodies when RIA were performed [14], implying that Ts IV either shares common epitopes with Ts VII or that the Ts IV preparation contained residual contamination by Ts VII (0.01% w/w) or Ts VIII (1% w/w). To eliminate the second of these possibilities, the Ts IV preparation was subjected to an additional immunoaffinity chromatography step on a column made with the IgG raised against the β-toxin Ts VII [14]. After filtration through this column, the Ts IV preparation was unable to displace [^{125}I]Css II from its binding site but was still able to compete with [^{125}I]AaH II with a $K_{0.5}$ of 3×10^{-7} M.

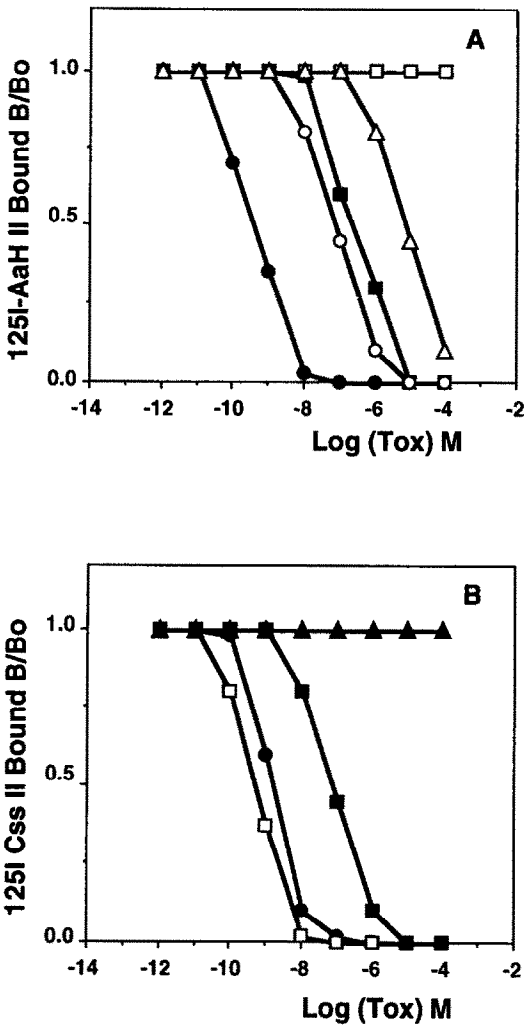

Fig. 3. Competition binding to rat brain synaptosomal fraction. (A) Competition experiments between the α-toxin, [^{125}I]AaH II (●), and Ts III (△), Ts IV (■), Ts V (○) and Ts VIII (□). (B) Competition experiments between the β-toxin, [^{125}I]Css II (●), and Ts VIII (□), Ts IV before (■) and Ts IV after (▲) immunoaffinity chromatography on an anti-Ts VII-IGg protein A–Sepharose column. Rat brain synaptosomal fraction (0.4 mg/ml) was incubated for 30 min at 37°C, with the α-toxin, [^{125}I]AaH II (0.2 nM), or the β-toxin, [^{125}I]Css II (0.1 nM), and concentrations of toxins. B_o is the binding of either [^{125}I]AaH II and [^{125}I]Css II in the absence of toxins, and B is the binding in the presence of the indicated concentrations of toxins. Non-specific binding in the presence of an excess of unlabelled toxin (1,000-times excess) has been subtracted. The incubation buffer used for competition with β-toxin [14] was: 25 mM HEPES, 10 mM glucose, 154 mM choline, 5.4 mM potassium chloride, 0.1% bovine serum albumin, pH 7.2. 1.8 mM calcium chloride and 1 mM magnesium sulphate were added to the incubation medium for competition with α-toxin, as both cations are inhibitors of β-toxin fixation [14]. Pellets were obtained by centrifugation (11,000 × *g* for 5 min), washed twice with cold buffer and the bound radioactivity counted in a γ-spectrophotometer (Packard).

Its s.c. toxicity was unchanged. Ts VIII was defined without ambiguity as a β-toxin: it competed for site 4 of the Na^+ channel with [^{125}I]Css II and not for site 3 with [^{125}I]AaH II. In spite of similar amino acid compositions, Ts VIII exhibited an affinity ($K_{0.5}$ of 5×10^{-10} M) for its receptor 100-times lower than that of the potent β-toxin, Ts VII.

4. Discussion

On the basis of their amino acid compositions alone, it is quite difficult to identify with certainty the Tityustoxin described by Diniz or toxin IV-5 described by Possani et al. [1,2] as Ts III, Ts IV or Ts V (this study). However, Ts IV (the most abundant toxin and the most active by s.c. injection) could be Tityustoxin. The pharmacological characterisation of Ts IV shows that it is an α-type toxin. The presumed residual contamination of the β-type toxin, eliminated by immunoaffinity chromatography, was undetectable by conventional methods of protein chemical analysis because its concentration was low. However, this contamination was very significant for pharmacological characterization since the β-type effect could be observed at concentrations of 100 nM. Probably, Ts III and Ts V are also α-toxins slighly contaminated by β-type toxin(s). Ts VIII could be a denaturated form of Ts VII. Authors who have studied *T. serrulatus* venom have demonstrated the complexity of the last fraction (S4) obtained by filtration and the polymorphism of its components [1,2,4]. Possani et al. found that toxin IV-6 was identical to toxin IV-5 up to residue number 30 and explained the difference in chromatographic behaviour by deamination of an Asn or a Gln of toxin IV-5 [2]. From this work, it can now be speculated whether different post-translational processing of the same precursor leads to the three mature peptides which vary only in their C-terminal sequence. The retention times observed for Ts III, Ts IV and Ts V on a cationic exchanger and their amino acid compositions differing by their Lys content are consistent with this scheme. These differences in amino acid sequence should be responsible for the different affinity and toxicity values determined in the biological experiments.

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