

Bibrotoxin, a novel member of the endothelin/sarafotoxin peptide family, from the venom of the burrowing asp *Atractaspis bibroni*

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A new member of the endothelin/sarafotoxin family of vasoconstrictor peptides, bibrotoxin (BTX), was isolated from the venom of the burrowing asp *Atractaspis bibroni* by reversed-phase FPLC. The amino acid sequence of BTX differs from SRTX-b in the substitution Ala¹ instead of Lys¹, which suggests that it represents the peptide isoform of *Atractaspis bibroni* corresponding to SRTX-b. BTX competed for [¹²⁵I]ET-1 binding to human ET_B-type receptor with a K_i of 3.2×10^{-9} M compared to 4.2×10^{-9} M for SRTX-b. In rat thorax aorta BTX induced vasoconstrictions with a threshold concentration of 3×10^{-8} M compared to 1×10^{-9} for ET-1.

Sarafotoxin; Endothelin; Snake venom; *Atractaspis bibroni*

1. INTRODUCTION

The peptides of the mammalian endothelin family [1,2] and of the sarafotoxin family from the venom of the burrowing asp *Atractaspis engaddensis* [3,4] are very similar in structure. The comparative analysis of the receptor binding and the vasoconstrictor activity of the natural isotoxins [5,6], of synthetic analogues [7] and of peptide fragments [8] has contributed to the understanding of the structure–function relationship of these peptides. The endothelins ET-1 and -2, like their snake counterparts the sarafotoxins SRTX-a and -b, are strong vasoconstrictors, that bind to and act through the same receptors in vascular and brain tissue [5,9,10].

While the sequence of the endothelins with the exception of mouse ET-2, i.e. vasointestinal contractor (VIC) [11], is highly conserved among mammals from mice to humans, nothing is known about the presence of sarafotoxin-related peptides in other reptiles, either in their venom or in their circulatory system. Neither is it known, if these peptides are synthesized as pro-forms (big-sarafotoxin) that are subsequently processed by converting enzymes.

In this paper we report the purification and characterisation of a novel peptide of the ET/SRTX family of peptides from the venom of the burrowing asp *Atrac-*

aspis bibroni, a South African relative of the Middle Eastern *A. engaddensis*.

2. EXPERIMENTAL

ET-1, ET-2, ET-3 and SRTX-b were purchased from Bachem (Heidelberg, Germany), dissolved at 0.1 mM and stored at -20°C . A rabbit polyclonal antibody to ET-1 was obtained after immunisation with ET-1 coupled to keyhole limpet hemocyanin using glutaraldehyde; the IgG fraction was isolated by Protein A-affinity chromatography and antibodies crossreacting with bigET-1 were removed by chromatography on bigET-Sepharose.

Specimens of *Atractaspis bibroni* were captured in Natal and were held with authority from the Natal Parks Board. The venom from six of the snakes was collected by having them strike laterally through a layer of parafilm stretched over a plastic ring. Droplets of venom that collected on the distal surface of the parafilm were rinsed with distilled water into sample tubes, freeze-dried and stored at -80°C until further use. Lyophilized venom was reconstituted with water and, after removal of particulate material, applied to a reversed phase FPLC column (PepRPC HR 5/5; Pharmacia). The column was eluted with a gradient from 0% to 50% buffer B (buffer A: 0.1% TFA/H₂O; buffer B: 0.085% TFA/acetonitrile) at 1 ml/min.

Venom fractions obtained by reversed-phase FPLC were analysed by coating aliquots to microplate wells. These were blocked with PBS/0.05% Tween 20 and developed with ET-1 antibodies that cross-reacted with SRTXb and peroxidase-labeled goat anti-rabbit IgG (Sigma, Deisenhofen, Germany).

Samples were analysed by RP-HPLC (Superspher RP-18, 4 μm , (250 \times 4) mm, MZ-Analysentechnik GmbH, Mainz, Germany) using a gradient from 0% to 50% buffer B (buffer A: 0.1% TFA/H₂O; buffer B: 0.085% TFA/acetonitrile) at 1 ml/min. Elution was monitored at 214 and 280 nm.

For the functional analysis of the fractions obtained from the venom by reversed-phase FPLC, aliquots were tested in a competitive radio receptor assay using membranes of BHK cells stably transfected with cloned human ET_B-type receptor (unpublished results and [12]) and (3-[¹²⁵I]iodotyrosyl) endothelin-1 ([¹²⁵I]ET-1; 2000 Ci/mmol \sim 74 TBq/mmol, Amersham) at a final concentration of 15 pM. Assays

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Abbreviations. ET, endothelin; SRTX, sarafotoxin; BTX, bibrotoxin; VIC, vasointestinal contractor.

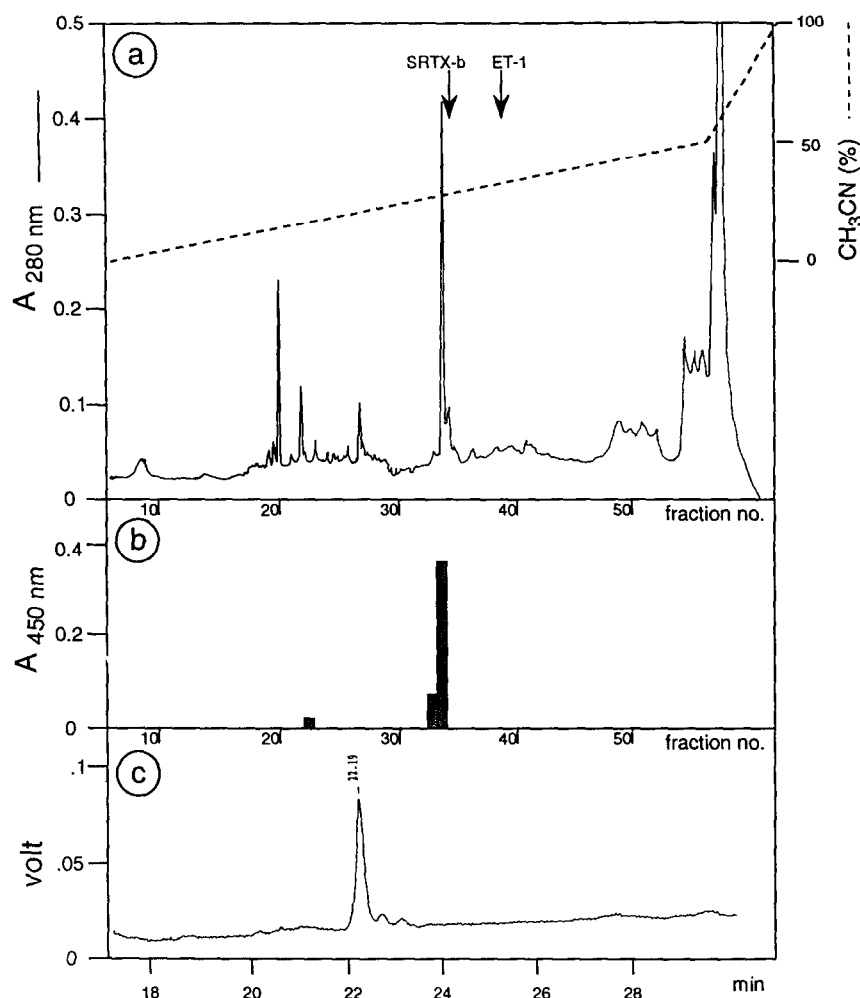


Fig. 1. Analysis of *A. bibroni* toxin by reversed-phase FPLC and identification of bibrotoxin by ELISA and HPLC. Reconstituted toxin was equilibrated with $H_2O/0.1$ TFA, applied to a FPLC PepRPC HR 5/5 column and eluted exactly as described under Experimental Procedures (a); retention times of SRTX-b and ET-1 are indicated by arrows. Fractions of 1 ml were collected and aliquots of $10\ \mu l$ of each fraction were coated to microtiter wells, which were developed as described under Experimental Procedures (b). $60\ \mu l$ of fraction 35 were analyzed by HPLC as described under Experimental Procedures (c).

were stopped by filtration on glass fiber filters (GF 25, Schleicher & Schüll) followed by three rapid washes with 0.5 ml of buffer. Non-specific binding was determined in the presence of unlabeled endothelin-1 (ET-1) at a concentration of 10^{-7} M. Competition binding assays were performed using varying concentrations of competitors as indicated in the text. Mathematical analysis of binding data was carried out using modified versions of the EBDA/LIGAND programs [13,14].

Sequence analysis of pyridylethylated reduced as well as untreated peptide was done using a model 477A protein sequencer with an on-line model 120A PTH analyzer (Applied Biosystems).

The vasoactive effect of bibrotoxin (BTX) was examined using isolated rings of rat thoracic aortae. Male Wistar rats (250–300 g, Schering SPF) were killed by carbon dioxide inhalation. The thoracic aorta was prepared and the isometric tension was recorded essentially as described elsewhere [15]. Briefly, the endothelium was removed by gently rubbing the internal surface with a watchmaker's forceps. The rings were mounted under 2.0 g resting tension in 10 ml organ chambers (Schuler, Hugo-Sachs, Germany) containing physiological saline solution. The tissues were allowed to equilibrate for 60 min before being contracted by addition of 25 mM KCl and, following the wash out, with phenylephrine ($1\ \mu M$). The lack of acetylcholine (ACh)-

induced ($1\ \mu M$) relaxation was taken as an indication of endothelium removal (E^-). The de-endothelialized rings were contracted with ET-1, SRTX-b, and BTX at the final concentrations indicated.

3. RESULTS AND DISCUSSION

Venom obtained from individual specimens of *A. bibroni* revealed different compositions when analysed by reversed-phase chromatography, a phenomenon commonly observed in the analysis of snake venoms [16]. In the representative sample shown (Fig. 1a and b) ET-like immunoreactivity was found in a single fraction, which eluted at 27% CH_3CN/TFA , corresponding to the retention time of SRTX-b in this system; ET-1 eluted at 33%. The same fraction that contained the material reacting with the antibody, specifically competed for binding of $[^{125}I]ET-1$ to membranes of BHK cells expressing human ET_B -type receptor (not shown).

ET-1	Cys	Ser	Cys	Ser	Ser	Leu	Met	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-2	Cys	Ser	Cys	Ser	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
VIC	Cys	Ser	Cys	Asn	Ser	Trp	Leu	Asp	Lys	Glu	Cys	Val	Tyr	Phe	Cys	His	Leu	Asp	Ile	Ile	Trp
ET-3	Cys	Thr	Cys	Phe	Thr	Tyr	Lys	Asp	Lys	Glu	Cys	Val	Tyr	Tyr	Cys	His	Leu	Asp	Ile	Ile	Trp
SRTX-a	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRTX-b	Cys	Ser	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Tyr	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRTX-c	Cys	Thr	Cys	Asn	Asp	Met	Thr	Asp	Glu	Glu	Cys	Leu	Asn	Phe	Cys	His	Gln	Asp	Val	Ile	Trp
SRTX-d	Cys	Thr	Cys	Lys	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Tyr	Phe	Cys	His	Gln	Asp	Ile	Ile	Trp
Bibrototoxin	Cys	Ser	Cys	Ala	Asp	Met	Thr	Asp	Lys	Glu	Cys	Leu	Tyr	Phe	Cys	His	Gln	Asp	Val	Ile	Trp

Fig. 2. Sequence of bibrotoxin in comparison to the endothelins and sarafotoxins. Amino acids common to all peptides of this family are boxed with rectangular corners, those shared by either the mammalian endothelins or the sarafotoxin family are boxed with round corners. The substitution that is unique to bibrotoxin (Ala^4) is set in bold and those amino acids that are shared by the most active members of each family (ET-1 and SRTX-b) are encircled.

This fraction contained a homogeneous peptide, as determined by HPLC analysis (Fig. 1c).

Two aliquots of this peptide were used to determine its sequence; one was reduced and pyridylethylated before both were sequenced. The sequence thus determined is highly homologous to that of SRTX-b (Fig. 2) with the only difference being the substitution of Ala^4 instead of Lys^4 . This exchange appears to be less important for its functional activity, because it is the least conserved position with five different amino acids known in the nine peptides of this family. Moreover, it shares Ser^2 with the sarafotoxins SRTX-a and -b as well as the endothelins-1 and -2, all of which are potent vasoconstrictors. It has been pointed out that this position may be important for the potency of these peptides in comparison to ET-3 and SRTX-c and -d that have Thr^2 and exhibit poor activity [5]. Besides the ten amino acids that are identical in all peptides of this family, the novel peptide shares the six substitutions that are identical within the sarafotoxins but are different from the endothelins.

In a competitive binding assay using membranes of BHK cells stably transfected with human ET_B -type receptor, very similar competition curves were found for ET-1, SRTX-b and BTX-b (Fig. 3). Non-linear regression analysis [14] of the data yielded a K_i of 3.2×10^{-9} M for BTX-b compared with 4.2×10^{-9} M for SRTX-b.

ET-1, BTX and SRTX-b contracted rat thoracic aortic preparations without endothelium in a concentration-dependent manner (Fig. 4). The potency order was: $\text{ET-1} \geq \text{SRTX}$. BTX had a threshold concentration of 3×10^{-8} M in denuded rings. Higher concentrations of BTX than indicated could not be tested due to the limited availability of this peptide. The vehicle elicited no vasoactive effect.

In summary, this novel peptide clearly is a member of the snake ET-related peptides and most likely represents the *A. bibroni* homologue of SRTX-b. Thus in analogy the name bibrotoxin(-b) (BTX) is proposed for this peptide. It is presently not known whether other bibrotoxins besides BTX exist, but a molecular biology approach using oligonucleotides derived from this sequence should soon answer this question. It is to be expected that a precursor of bibrotoxin (big-bibrotoxin) as it is present in mammals (big-endothelin) can be identified in these snakes. Given the specialized function of their venom glands it may be possible to identify and

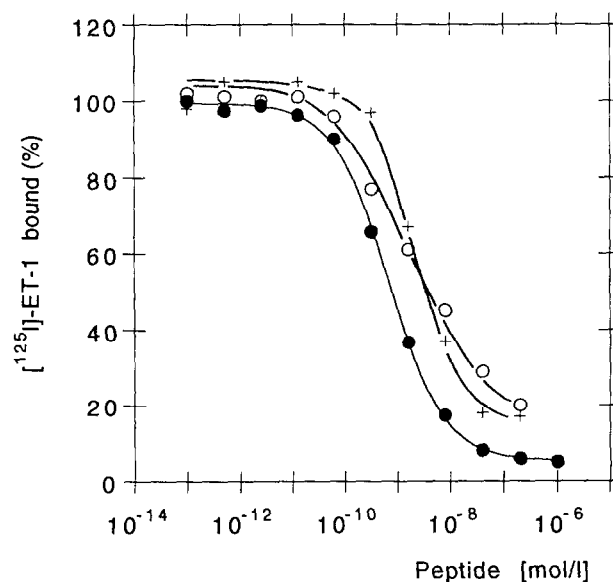


Fig. 3. Competition of $[^{125}\text{I}]\text{ET-1}$ binding to ET-R_B by ET-1, SRTX-b and BTX. Competitive binding assays were carried out as described under Experimental Procedures using ET-1 (●), SRTX-b (○), and BTX (+) at the final concentrations indicated.

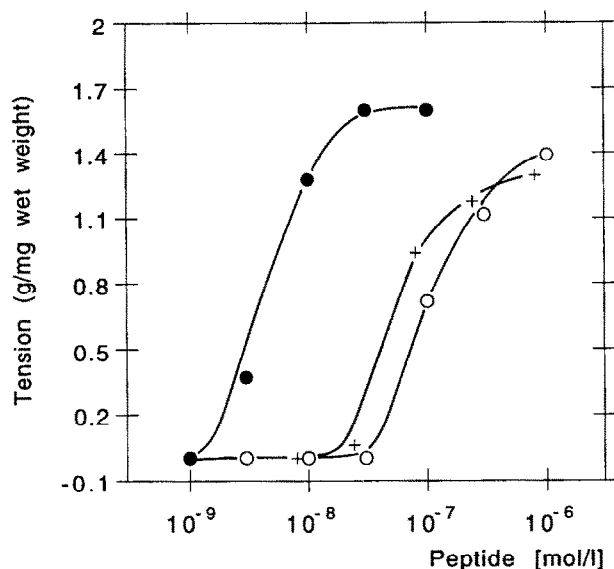


Fig. 4. Vasoactive effects of BTX in comparison to ET-1 and SRTX-b. Contractions of rings of rat aorta induced by ET-1 (●), SRTX-b (○), and BTX (+) in the absence of endothelium. Each point represents an individual experiment.

isolate the converting enzyme which in mammals has proven to be so elusive.

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