

Mônica A. Coronado,^a
Dessislava Georgieva,^b Friedrich
Buck,^c Azat H. Gabdoulkhakov,^d
Anwar Ullah,^a Patrick J.
Spencer,^e Raghuvir K. Arni^a and
Christian Betzel^{b*}

^aCentro Multiusuário de Inovação Biomolecular, Departamento de Física, Universidade Estadual Paulista (UNESP), São José do Rio Preto-SP 15054-000, Brazil, ^bLaboratory for Structural Biology of Infection and Inflammation, University of Hamburg, c/o DESY, Notkestrasse 85, 22603 Hamburg, Germany, ^cInstitute of Clinical Chemistry, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany, ^dInstitute of Crystallography, Russian Academy of Sciences, 59 Leninsky Prospect, Moscow 119333, Russian Federation, and ^eCentro de Biotecnologia, Instituto de Pesquisas Energéticas e Nucleares—CNEN/SP, Avenida Professor Lineu Prestes 2242, São Paulo-SP 05508-000, Brazil

Correspondence e-mail:
christian.betzel@uni-hamburg.de

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Purification, crystallization and preliminary X-ray diffraction analysis of crotoamine, a myotoxic polypeptide from the Brazilian snake *Crotalus durissus terrificus*

Crotoamine, a highly basic myotoxic polypeptide (molecular mass 4881 Da) isolated from the venom of the Brazilian rattlesnake *Crotalus durissus terrificus*, causes skeletal muscle contraction and spasms, affects the functioning of voltage-sensitive sodium channels by inducing sodium influx and possesses antitumour activity, suggesting potential pharmaceutical applications. Crotoamine was purified from *C. durissus terrificus* venom; the crystals diffracted to 1.9 Å resolution and belonged to the orthorhombic space group $I2_12_1$ or $I222$, with unit-cell parameters $a = 67.75$, $b = 74.4$, $c = 81.01$ Å. The self-rotation function indicated that the asymmetric unit contained three molecules. However, structure determination by molecular replacement using NMR-determined coordinates was unsuccessful and a search for potential derivatives has been initiated.

1. Introduction

Crotoamine induces spastic paralysis and myonecrosis (Katagiri *et al.*, 1998), binding strongly to excitable membranes, and causes the contraction of skeletal muscle, resulting in rapid lysis of the sarcolemma, myofibril clumping and hypercontraction of sarcomeres (Matavel *et al.*, 1998). This toxin induces skeletal muscle spasms (Oguiura *et al.*, 2005) and interferes with the functioning of voltage-sensitive sodium channels of the skeletal muscle sarcolemma, leading to rapid sodium influx. Crotoamine also causes depolarization and contraction of skeletal muscle attributed to the inhibition of voltage-gated potassium channels (Peigneur *et al.*, 2012). More recently, it has been demonstrated that crotoamine targets tumour tissue *in vivo* and triggers a lethal calcium-dependent pathway in cultured cells (Nascimento *et al.*, 2011).

Crotoamine (GenBank accession code P01475), a multifunctional small (molecular mass 4881 Da) and highly basic (pI = 10.3; Giglio, 1975) protein isolated from the venom of *Crotalus durissus terrificus* (Gonçalves & Polson, 1947; Laure, 1975), belongs to a class of closely related polypeptide myotoxins which are stabilized by three disulfide bridges. Crotoamine is unglycosylated, rich in arginine and lysine residues (Boni-Mitake *et al.*, 2001) and shares a relatively low sequence identity of 23% with human β -defensins. Multiple sequence alignments indicated partial conservation of the β -defensin fold (Schibli *et al.*, 2002). The relatively highly positive potential surface charge allows us to hypothesize that it can interact electrostatically with the negative surface of membranes, causing local disruption and thereby inducing leakage of ions.

Relatively large and well diffracting crystals of crotoamine have been obtained. However, exhaustive attempts to solve the crystal structure using models based on two independently determined NMR structures of crotoamine (Nicastro *et al.*, 2003; Fadel *et al.*, 2005) have not been successful, probably owing to inherent flexibility or disorder. Thus, since neither the crystal structures nor the multimer interactions of these peptides have been determined, a search for suitable derivatives has been initiated.

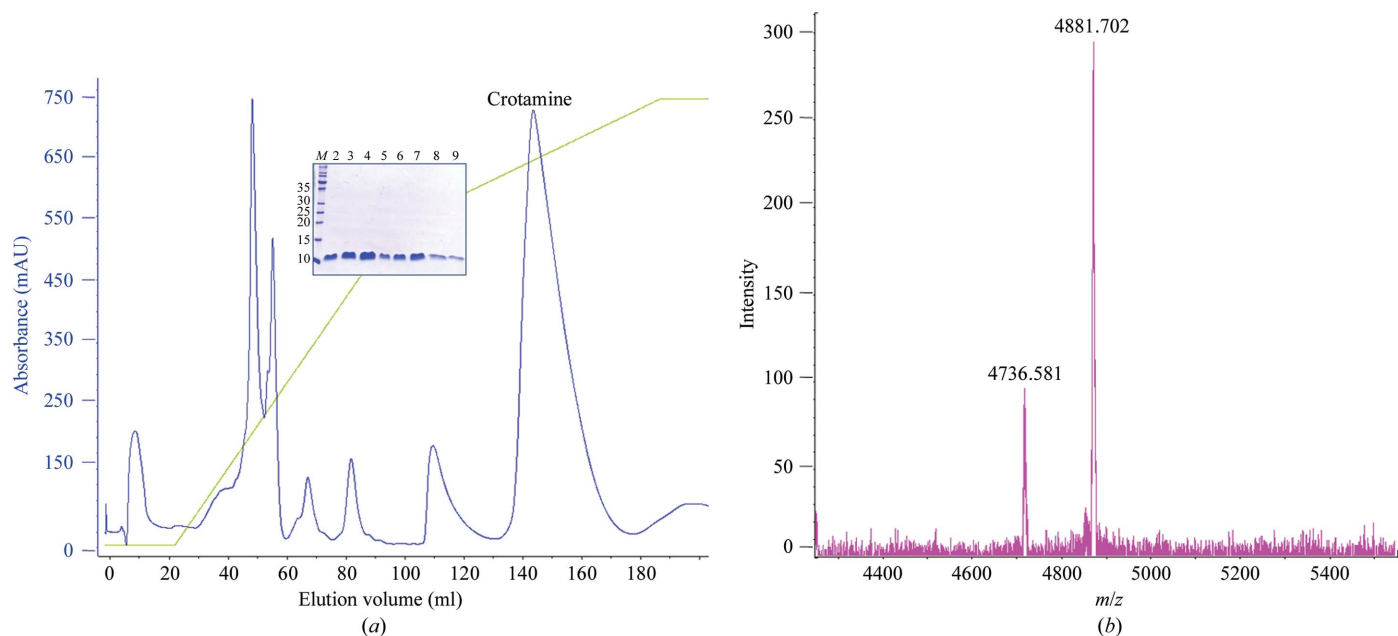


Figure 1
 (a) Purification of crotonamine by cation-exchange chromatography (Mono S HR 10/10). Inset, Coomassie-stained 12% SDS-PAGE gel of crotonamine. Lane M, molecular-mass markers (labelled in kDa); lanes 2–9, pooled purified crotonamine at different concentrations. (b) Representative MALDI-TOF spectrum obtained from crotonamine.

2. Materials and methods

2.1. Venom collection and purification

Since the content of crotonamine present in the venom of *C. durissus terrificus* varies according to geographical location, crude venom was obtained from a number of different sources. The venom obtained from CEVAP, Botucatu, Brazil contained a significantly greater amount of crotonamine (approximately 15% by weight). 150 mg desiccated crude venom was dissolved in 2 ml 0.05 M acetic acid pH 5 and centrifuged at 10 000g for 10 min. The clear supernatant was applied onto a Mono S HR 10/10 column (Amersham Biosciences; Fig. 1a) previously equilibrated with the same buffer, the column was washed at a flow rate of 60 ml h⁻¹ until the baseline stabilized and the bound fractions were eluted with the previously described solution additionally containing 1.0 M NaCl.

The purity of the protein was confirmed using SDS-PAGE gels (12%) as described by Laemmli (1970). Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Fig. 1a, inset).



Figure 2
 Native crystals of crotonamine with approximate dimensions of 0.5 × 0.25 × 0.1 mm.

Protein concentrations were determined by calculating the theoretical extinction coefficient and absorption was measured at 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

The molecular mass was confirmed by mass spectroscopy (MALDI-TOF) on an UltrafleXtreme instrument (Bruker Daltonics, Bremen, Germany) and the sequence of the first ten N-terminal amino acids was confirmed by Edman degradation (476A protein sequencer, Applied Biosystems; Fig. 1b).

2.2. Crystallization

For crystallization experiments, crotonamine was concentrated to 22 mg ml⁻¹ in ultrapure water using microconcentrators (Amicon Ultra-4, Ultracel membrane). *In situ* dynamic light-scattering (DLS) measurements were carried out at 293 K at the same concentration using a SpectroLIGHT 500 system (Nabitek, Germany; Garcia-Caballero *et al.*, 2011). DLS confirmed the presence of a single monodisperse population.

Crystallization conditions for crotonamine were screened using the vapour-diffusion method (McPherson, 1999). A total of 384 crystallization conditions based on the commercially available JCSG+, ComPAS, Classics and Cryos Suites (NeXtal, Qiagen) were screened using a Honeybee 961 dispensing robot (Zinsser Analytic GmbH, Frankfurt, Germany) at 293 K in 96-well crystallization plates (NeXtal QIA1 µplates, Qiagen) using the sitting-drop vapour-diffusion method. A 300 nl droplet of protein at approximately 10 mg ml⁻¹ in buffer was mixed with the same volume of reservoir solution and equilibrated against 35 µl reservoir solution. The initial crystals obtained were relatively small; in order to increase their quality and size, they were reproduced manually using the hanging-drop vapour-diffusion method in Linbro 24-well plates. Diffraction-quality crystals with approximate dimensions of 0.5 × 0.25 × 0.1 mm were obtained after 2 d when a 1 µl protein droplet was mixed with an equal volume of reservoir solution consisting of 0.2 M sodium thiocyanate, 1.9 M ammonium sulfate pH 6.1 (Fig. 2).

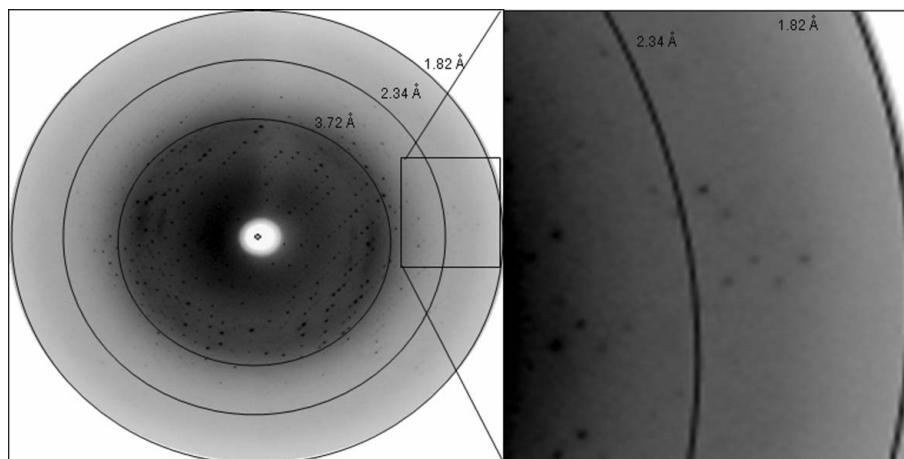


Figure 3
X-ray diffraction pattern of croptamine: concentric rings indicate resolution ranges and the high-resolution diffraction pattern is magnified.

Table 1

Summary of data-collection and crystal parameters.

Values in parentheses are for the highest resolution shell.

Radiation source	X13 Consortium Beamline, DESY/HASYLAB
Detector	MAR CCD
Wavelength (Å)	0.8123
Space group	$I222$ or $I2_12_12_1$
Unit-cell parameters (Å)	$a = 67.75$, $b = 74.40$, $c = 81.01$
V_M (Å ³ Da ⁻¹)	3.54
Solvent content (%)	65.2
Resolution range (Å)	18.7–1.9 (1.90–1.85)
Data completeness (%)	94.8 (76.1)
R_{merge}^\dagger (%)	3.6 (48.9)
$\langle I/\sigma(I) \rangle$	34.7 (3.6)

$^\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $\langle I(hkl) \rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl .

2.3. X-ray data collection

For diffraction data collection, a single crystal was flash-cooled after soaking it in reservoir solution that additionally contained 20% glycerol prior to data collection. X-ray diffraction data were collected on the X13 Consortium Beamline at DESY/HASYLAB. The wavelength of the radiation source was set to 0.8123 Å and a MAR CCD detector was used to record the X-ray diffraction intensities as 256 images with an oscillation range of 1° per image (Fig. 3). The raw intensities were indexed, integrated and scaled using the program *MOSFLM* (Leslie & Powell, 2007) from the *CCP4* suite (Winn *et al.*, 2011). Details of the data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

The molecular mass of the purified croptamine was determined to be 4881.70 Da and the presence of an isoform (with a molecular mass of 4736.58 Da) was observed (Fig. 1*b*). The croptamine crystals (Fig. 2) diffracted X-rays to 1.8 Å resolution (Fig. 3). Processing of the diffraction data resulted in an R_{merge} of 4.3% and a completeness of 97.05%. Examination of the systematic absences indicated that the crystals belonged to the enantiomorphic space groups $I2_12_12_1$ or $I222$, with unit-cell parameters $a = 67.75$, $b = 74.40$, $c = 81.01$ Å. Based on the results of the self-rotation function, the Matthews coefficient (Matthews, 1968) was calculated to be 3.54 Å³ Da⁻¹ assuming the presence of three molecules of croptamine in the asymmetric unit, which corresponds to a solvent content of 65.2%. Since both structure

determination by molecular replacement using various models based on the independently determined NMR atomic coordinates (Nicastro *et al.*, 2003; Fadel *et al.*, 2005) with the program *MOLREP* (Vagin & Teplyakov, 2010) and attempts to use the anomalous sulfur signals were not successful, a search for suitable derivatives has been initiated.

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