Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of bucain, a novel toxin from the Malayan krait *Bungarus candidus*

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Received 25 February 2002
Accepted 19 June 2002

Bucain is a three-finger toxin, structurally homologous to snake-venom muscarinic toxins, from the venom of the Malayan krait *Bungarus candidus*. These proteins have molecular masses of approximately 6000–8000 Da and encompass the potent curaremimetic neurotoxins which confer lethality to *Elapidae* and *Hydrophidae* venoms. Bucain was crystallized in two crystal forms by the hanging-drop vapour-diffusion technique in 0.1 *M* sodium citrate pH 5.6, 15% PEG 4000 and 0.15 *M* ammonium acetate. Form I crystals belong to the monoclinic system space group *C*2, with unit-cell parameters a = 93.73, b = 49.02, c = 74.09 Å, β = 111.32°, and diffract to a nominal resolution of 1.61 Å. Form II crystals also belong to the space group *C*2, with unit-cell parameters a = 165.04, b = 49.44, c = 127.60 Å, β = 125.55°, and diffract to a nominal resolution of 2.78 Å. The self-rotation function indicates the presence of four and eight molecules in the crystallographic asymmetric unit of the form I and form II crystals, respectively. Attempts to solve these structures by molecular-replacement methods have not been successful and a heavy-atom derivative search has been initiated.

1. Introduction

Snake venoms are complex mixtures of proteins and polypeptides. Venom from a single species may contain several hundred different proteins, which may however be classified into a small number of superfamilies. One such superfamily, the three-finger family of toxins, has a molecular mass in the approximate range 6000–8000 Da and encompasses the potent curaremimetic neurotoxins which confer lethality to *Elapidae* and *Hydrophidae* venoms. Other members include muscarinic toxins with selectivity towards distinct types of muscarinic receptors (Jerusalinsky & Harvey, 1994), fasciculins that inhibit acetylcholinesterase (Cervenansky *et al.*, 1991), calciseptins that block the L-type calcium channels (De Weille *et al.*, 1991; Albrand *et al.*, 1995), cardiotoxins (cytotoxins) that exert their toxicity by forming pores in cell membranes (Bilwes *et al.*, 1994) and dendroaspins, which are antagonists of various cell-adhesion processes (McDowell *et al.*, 1992). Three-finger toxins derive their name from their unique tertiary fold which adopts a leaf-like shape comprising of three adjacent loops ('fingers') forming a large and flat β-pleated sheet that emerges from a small globular core confined by four conserved disulfide bridges (Endo & Tamiya, 1991; Menez, 1998; Tsetlin, 1999). Certain classes of neurotoxins, such as the long-chain α-neurotoxins (*i.e.* α-bungarotoxin from *B. multicinctus*) and the neuronal κ-bungarotoxin (*B. multicinctus*) also have an additional (fifth) disulfide bridge that is located in the middle loop (loop II; Endo & Tamiya, 1991; Menez, 1998; Tsetlin, 1999). This fifth bridge is located in the first loop (loop I) in a group of poorly characterized toxins called 'weak toxins' which are typically of a lower order of toxicity (~50–1000 times less potent) compared with prototypical α-neurotoxins (Mebs & Claus, 1991). Interestingly, the three-fingered motif is not uniquely adopted by snake-venom toxins; it is also associated with proteins from non-venom sources such as the *Ly-6* family of cell-surface accessory proteins (Fleming *et al.*, 1993; Ploug & Ellis, 1994; Gumley *et al.*, 1995) and *lynx 1*, which has been identified as a novel protein modulator of neuronal nicotinic acetylcholine receptors (Miwa *et al.*, 1999). We have recently purified and characterized two three-finger toxins from *B. candidus* venom that are structurally related to weak toxins. The crystal structure of bucandin (Kuhn *et al.*, 2000), a novel toxin with a yet undetermined molecular target, has been described. The other, candoxin, is a novel antagonist of nicotinic acetylcholine receptors (Nirthanan *et al.*, manuscript in preparation). We now describe the crystallization and preliminary diffraction data of bucain, a three-finger toxin structurally homologous to snake-

venom muscarinic toxins, from the venom of the Malayan krait *B. candidus*.

2. Materials and methods

2.1. Materials

Lyophilized *B. candidus* venom (BCV) was obtained from Venom Supplies (Tanunda, SA, Australia). The pre-packed columns Superdex 30 and Jupiter C18 were purchased from Pharmacia Biotech (Sweden) and from Phenomenex (CA, USA), respectively. The following chemicals were purchased from the sources indicated: reagents for N-terminal sequencing from Applied Biosystems, Foster City, CA, USA, acetonitrile from Fisher Scientific, Fair Lawn, NJ, USA, 4-vinlypyridine and trifluoroacetic acid (TFA) from Fluka Chemika–Biochemika, Buchs, Switzerland. All other chemicals were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

2.2. Purification

Whole lyophilized venom (150 mg) was dissolved in 1 ml of MilliQ water, centrifuged at 4500*g* for 20 min and the supernatant fractionated based on the molecular weight of the components on a Superdex 30 fast protein liquid-chromatography (FPLC) column (1.6 × 60 cm). Tris–HCl buffer (50 m*M*, pH 7.5) at a flow rate of 2 ml min^{-1} was used as the eluent. The gel-filtration fraction containing bucain was further purified on a reverse-phase Jupiter C18 (0.2 × 25 cm) column as indicated in the legend to Fig. 1(*a*).

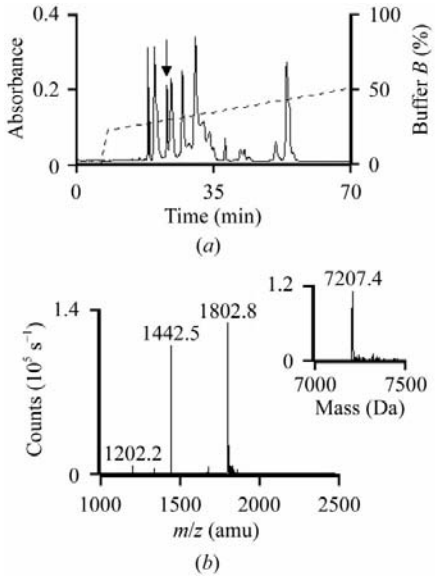

Figure 1
(*a*) Reverse-phase HPLC of pooled gel filtrations on a Jupiter C18 column using a linear gradient of buffer *B* (80% ACN in 0.1% TFA) at a flow rate of 2 ml min^{-1}. The peak indicated by the arrow contained bucain. (*b*) Electrospray ionization mass spectrum of bucain. The spectrum shows a series of multiply charged ions, corresponding to a single homogenous peptide of MW 7207.4 Da (inset, reconstructed spectrum).

2.3. Electrospray ionization mass spectrometry

Bucain was dissolved in 50% acetonitrile and 50% water and was subjected to electrospray ionization (ESI) mass spectrometry using a Perkin–Elmer Sciex API 300 triple-quadrupole instrument equipped with an ion-spray interface. The ion-spray voltage was set to 4600 V and the orifice voltage at 30 V. Nitrogen was used as curtain gas with a flow rate of 0.6 l min^{-1}, while compressed air was used as a nebulizer gas. The sample was infused into the mass spectrometer by flow injection at a flow rate of 50 µl min^{-1} using Shimadzu 10 AD pumps as the solvent-delivery system.

2.4. Determination of the N-terminal amino-acid sequence

Bucain was resuspended in 100 µl of the denaturant buffer (6.0 *M* guanidinium hydrochloride, 0.13 *M* Tris, 1 m*M* EDTA pH 8.0) containing 0.07 *M* β-mercaptoethanol. The solution was heated at 310 K for 2 h. Subsequently, a 1.5-fold molar excess (over sulfhydryl groups) of 4-vinylpyridine was added and incubated at room temperature. After 2 h, the sample was desalted by reversed-phase HPLC. Amino-terminal sequencing of the native and pyridylethylated protein was performed by automated Edman degradation using a Perkin–Elmer Applied Biosystems 494 pulsed-liquid-phase protein sequencer (Procize) with an on-line 785A PTH amino-acid analyzer.

2.5. Analysis of primary and three-dimensional structure of bucain

The amino-acid sequence of bucain was subjected to a similarity search using *BLAST* (Altschul *et al.*, 1990) and multiple sequence alignment carried out using *CLUSTALW* (Thompson *et al.*, 1994).

2.6. Crystallization and X-ray analysis

A lyophilized sample of bucain was dissolved in doubly distilled water at a concentration of 10 mg ml^{-1}. Crystallization was performed by the hanging-drop vapour-diffusion method using 24-well tissue-culture plates. Initial trials were carried out with Hampton Research Crystal Screen I. Typically, 1 µl drops of protein solution were mixed with an equal volume of the screening solution and equilibrated over 1 ml of the latter as reservoir solution. The conditions were refined and two single-crystal forms (~0.3 × 0.2 × 0.2 and 0.1 × 0.1 × 0.3 mm) were obtained when a 2 µl protein droplet was mixed with an equal volume of reservoir solution consisting of 0.1 *M* sodium citrate pH 5.6, 15% PEG 4000 and 0.15 *M* ammonium acetate (Fig. 2).

Table 1
X-ray diffraction data-collection statistics of the bucain crystals.

Values in parentheses are for the outermost resolution shell, 1.65–1.61 Å for form I and 2.84–2.78 Å for form II.

Form	I	II
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	*a* = 93.73, *b* = 49.02, *c* = 74.09, β = 111.32	*a* = 165.04, *b* = 49.44, *c* = 127.60, β = 125.55
Maximum resolution (Å)	1.61	2.78
Resolution of data set (Å)	35.0–1.61	14.0–2.78
No. of unique reflections	37350	19679
$R_{\rm merge}$† (%)	5.80	11.10
Completeness (%)	91.40 (50.40)	93.40 (83.50)
V_M‡ (Å^3 Da^{-1})	2.75	3.67
Solvent content (%)	54.92	66.25
No. of molecules per asymmetric unit	4	8
$\langle I/\sigma(I)\rangle$ (outermost shell)	23.4 (1.77)	7.0 (1.45)

† $R_{\rm merge} = \sum|I(h)_i - \langle I(h)\rangle|/\sum I(h)_i$, where $I(h)_i$ is the observed intensity of the *i*th measurement of reflection *h* and $\langle I(h)\rangle$ is the mean intensity of reflection *h* calculated after scaling. ‡ Matthews (1968).

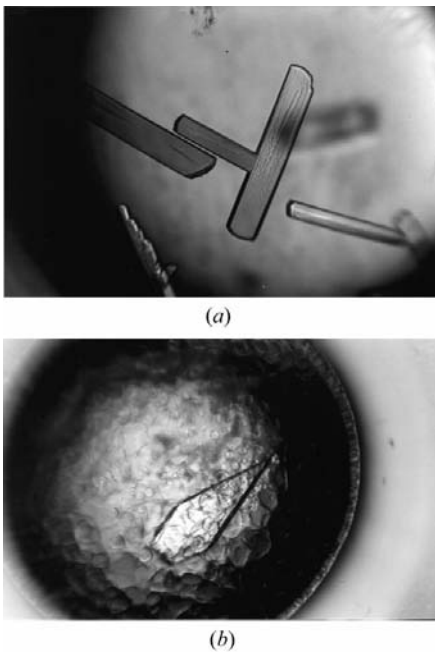

Figure 2
Photomicrograph of crystals used for X-ray diffraction experiments.

Both crystals forms of bucain were transferred to a mother-liquor solution containing 10% glycerol and were flash-frozen. X-ray diffraction data sets were collected at the crystallographic beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) at the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). The synchrotron-radiation source at LNLS was set to a wavelength of 1.54 Å. Two diffraction data sets were collected from 120 and 88 images, respectively, using the oscillation method. Individual frames consisted of an oscillation of 1° with the detector distance set to 110 and 200 mm, respectively. The crystals diffracted X-rays to nominal resolutions of 1.61 and 2.78 Å, respectively. Diffraction intensities were measured using a MAR345 imaging-plate detector and were reduced and processed using the *HKL* suite of programs (Otwinowski & Minor, 1997). Data-processing statistics are presented in Table 1.

3. Results

3.1. Isolation and purification

Bucain was purified to homogeneity by a two-step process involving gel filtration and reverse-phase HPLC as indicated in the legend to Fig. 1(*a*). Bucain has a molecular mass of 7207.4 (4) as determined by ESI mass spectrometry (Fig. 1*b*). It constitutes about 2–3% of the crude venom. Bucain was also found to be homogenous by ESI mass spectrometry. The complete amino-acid sequence of bucain is presented in Fig. 3(*a*).

3.2. Crystallization and data collection

Crystals forms I and II belong to space group *C*2, with unit-cell parameters a = 93.73, b = 49.02, c = 74.09 Å, β = 111.32° and a = 165.04, b = 49.44, c = 127.60 Å, β = 125.55°, respectively, and diffract to nominal resolutions of 1.61 and 2.78 Å, respectively; indeed, the completeness is quite low in the outer shell (Table 1). For the form I crystals, a complete data set (120°) was collected and the crystal was then translated and an additional data set (110°) was collected from the same crystal to provide a total of 330° of data which resulted in the high redundancy (Table 1).

The self-rotation functions carried out with the two data sets indicate the existence of four and eight molecules in the asymmetric unit of forms I and II, respectively.

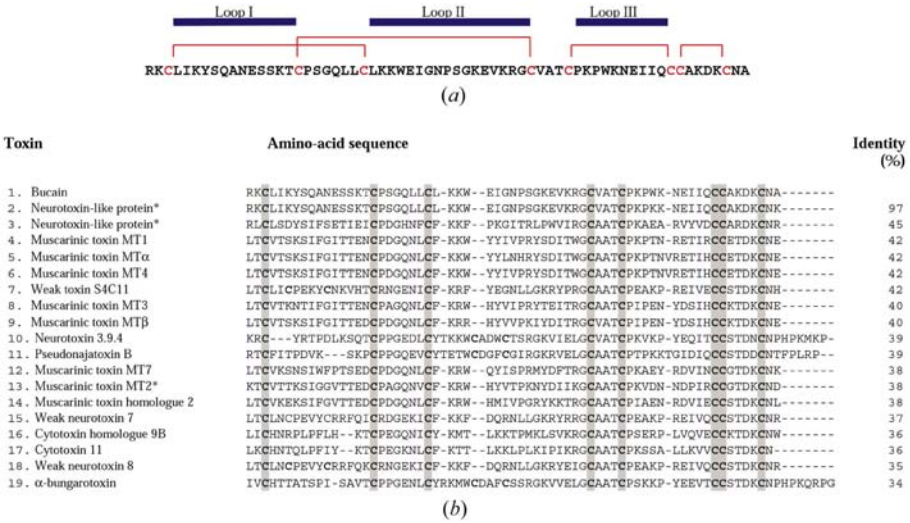

Figure 3
(*a*) The amino-acid sequence of bucain. Cysteine residues are marked in red. The four conserved disulfide linkages and the segments contributing to the three loops are outlined. (*b*) Comparison of the sequence of bucain. The sequence data were obtained from either SWISS-PROT or TREMBL databases. The conserved cysteine residues are highlighted. The percentage identity of each sequence to the sequence of bucain is also shown. The accession numbers and species names are as follows: 1, bucain (*B. candidus*); 2, Q9YGI8 (*B. multicinctus*); 3, Q9W717 (*Naja naja*); 4, P81030 (*Dendroaspis angusticeps*); 5, P80494 (*D. polylepis*); 6, Q9PSN1 (*D. angusticeps*); 7, P01400 (*N. melanoleuca*); 8, Q9PRH6 (*D. angusticeps*); 9, P80495 (*D. polylepis*); 10, P01383 (*N. melanoleuca*); 11, P13495 (*Pseudonaja textilis*); 12, P80970 (*D. angusticeps*); 13, P18328 (*D. angusticeps*); 14, P82463 (*N. naja kaouthia*); 15, P29181 (*N. naja*); 16, P24778 (*Hemachatus haemachatus*); 17, P01472 (*N. haje annulifera*); 18, P29182 (*N. naja*); 19, P01378 (*B. multicinctus*). Precursor proteins for which the amino-acid sequences were deduced from their respective cDNAs are marked with an asterisk.

Molecular-replacement studies were carried out with the program *AMoRe* (Navaza, 1994) using a number of models based on homology and using data in different resolution ranges. The 150 peaks with the highest correlation coefficients in the cross-rotation solutions were further examined using the translation search. However, no consistent solutions were obtained and a search for heavy-atom derivatives has been initiated to solve the structure.

The financial support of FAPESP, CNPq and FUNDUNESP is gratefully acknowledged. LW is the recipient of a FAPESP fellowship.

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