Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of a Kunitz-type inhibitor, textilinin-1 from *Pseudonaja textilis textilis*

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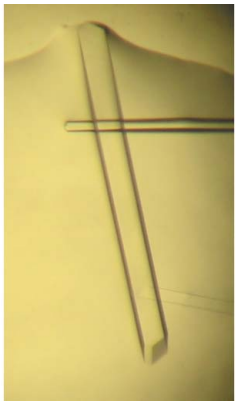
Received 24 February 2006
Accepted 23 May 2006



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Textilinin-1 (Txln-1), a Kunitz-type serine protease inhibitor, is a 59-amino-acid polypeptide isolated from the venom of the Australian Common Brown snake *Pseudonaja textilis textilis*. This molecule has been suggested as an alternative to aprotinin, also a Kunitz-type serine protease inhibitor, for use as an anti-bleeding agent in surgical procedures. Txln-1 shares only 47% amino-acid identity to aprotinin; however, six cysteine residues in the two peptides are in conserved locations. It is therefore expected that the overall fold of these molecules is similar but that they have contrasting surface features. Here, the crystallization of recombinant textilinin-1 (rTxln-1) as the free molecule and in complex with bovine trypsin (229 amino acids) is reported. Two organic solvents, phenol and 1,4-butanediol, were used as additives to facilitate the crystallization of free rTxln-1. Crystals of the rTxln-1–bovine trypsin complex diffracted to 2.0 Å resolution, while crystals of free rTxln-1 diffracted to 1.63 Å resolution.

1. Introduction

Venoms from snakes, cone snails, scorpions, spiders and sea anemones are cocktails of many small proteins or peptides with a range of biological activities (Lewis & Garcia, 2003). For example, cone snails have peptides that act as calcium or sodium channel blockers or bind to the nicotinic acetylcholine receptor (Nielsen *et al.*, 2000). Small proteins or peptides found in snake venoms include the PLA_2 phospholipases (Kwong *et al.*, 1989) and Kunitz-type serine protease inhibitors, such as the venom basic protease inhibitor (Chen *et al.*, 2001) and the textilinins (Masci *et al.*, 2000).

To date, six textilinins from *Pseudonaja textilis textilis*, the Australian Common Brown snake, have been identified by cloning from venom-gland cDNA (Masci *et al.*, 2000). All consist of ∼60 amino-acid residues. Their sequence identities range from 67 to 93%, with the identity between textilinin-1 (Txln-1) and textilinin-2 (Txln-2) being the highest. The greatest variation across the six sequences is observed in the segment between amino-acid residues 17 and 21, which is part of the so-called canonical loop and is the region that is most likely to make direct contact to the putative target serine proteases. All of the textilinins have a highly conserved serine/lysine propeptide cleavage site as well as the characteristic six-cysteine-residue motif common to Kunitz-type serine protease inhibitors. The 24-amino-acid propeptide sequence is hydrophobic, which suggests that it facilitates secretion of the protein prior to cleavage. Of the six textilinins that have been characterized, only Txln-1 and Txln-2 are effective in reducing blood loss in the mouse-tail model (Filippovich *et al.*, 2002). A sequence alignment of the textilinins shows that arginine in position 17 (the residue that binds in the P1 pocket in serine proteases) is common to Txln-1 and Txln-2, while the other four textilinins have an asparagine, lysine, glutamate or aspartate in this position.

Txln-1 has been suggested as an alternative to aprotinin (marketed as Trasylol) as an anti-bleeding agent during surgical procedures. It is believed that Txln-1's anti-bleeding property is a consequence of its ability to inhibit plasmin; it has a K_i value of 13.5 n*M* for this enzyme (Flight *et al.*, unpublished data). Txln-1 is also a potent inhibitor of trypsin, with a K_i value of 36 n*M*, and a moderate inhibitor of tissue kallikrein, with a K_i value of 3 μ*M* (Flight *et al.*, unpublished data).

No inhibitory activity was observed when the propeptide form of Txln-1 was used in the plasmin-inhibition assay, suggesting that this segment blocks the inhibitory site.

Txln-1 is an acidic molecule with a pI of 4.4 (Filippovich *et al.*, 2002), while aprotinin is basic with a pI of 8.9 (Gebhard *et al.*, 1986). The reason for this difference is that there are 11 negatively charged and seven positively charged amino acids in Txln-1, while in aprotinin there are only four negatively charged and ten positively charged amino acids. Surprisingly, only four of the charged amino acids are in identical positions in the two inhibitors. An important difference between the two inhibitors is that the amino acid that binds to the P1 pocket is lysine in aprotinin, while it is arginine in Txln-1. Overall, Txln-1 is one amino acid longer than aprotinin, having two extra amino acids at the amino-terminus but one fewer at the carboxy-terminus. Thus, the molecular surfaces of the two peptides are significantly different. In this report, we present preliminary crystallization data for the complex between bovine trypsin and recombinant Txln-1 and the crystallization of free recombinant Txln-1 (rTxln-1). This is the first report detailing the crystallization conditions for any of the textilinins.

2. Material and methods

2.1. Expression and purification of rTxln-1

rTxln-1 (accession No. AAK95519) was produced at BresaGen Ltd (Adelaide, South Australia). The cloned construct TVWT consisted of a proprietary N-terminal expression-enhancer sequence (TV), an internal tryptophan residue (W) and the 59-amino-acid mature Txln-1 sequence (T). The protein was expressed as inclusion bodies in *Escherichia coli* MM294 cells. The inclusion bodies were dissolved at an alkaline pH at a concentration of ∼2 mg ml^{-1}. Refolding of the fusion protein was initiated by lowering the pH to ∼9.5 with 1 *M* HCl. The progression of refolding was assessed by RP-HPLC. After ∼2 h, cleavage of the TVW moiety was initiated by the addition of *N*-chlorosuccinimide (Lischwe & Sung, 1977). The released and correctly folded rTxln-1 was purified by a combination of phenyl-Sepharose HP hydrophobic interaction chromatography and Q-Sepharose HP anion-exchange chromatography (Amersham Biosciences). The purified rTxln-1 containing fractions from the Q-Sepharose HP column were pooled and applied onto a Sephadex G25M column equilibrated with 25 m*M* Tris–HCl pH 7.8. The rTxln-1 was stored at 277 K in 25 m*M* Tris–HCl pH 7.8, 50 mg ml^{-1} mannitol and 0.01% Tween-20. In our laboratory, the purity was assessed by RP-HPLC to be >97%. Mass spectrometry gave a molecular weight of 6685 Da, which is within 1 Da of the expected value. Titration of a trypsin solution with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967) in the presence and absence of a sub-equimolar amount of rTxln-1 gave an estimate of the molar concentration of rTxln-1 which was in excellent agreement with the molar concentration calculated from the UV spectrum (using ε_{280} = 4560 M^{-1} cm^{-1}, estimated by the method of Gill & von Hippel, 1989).

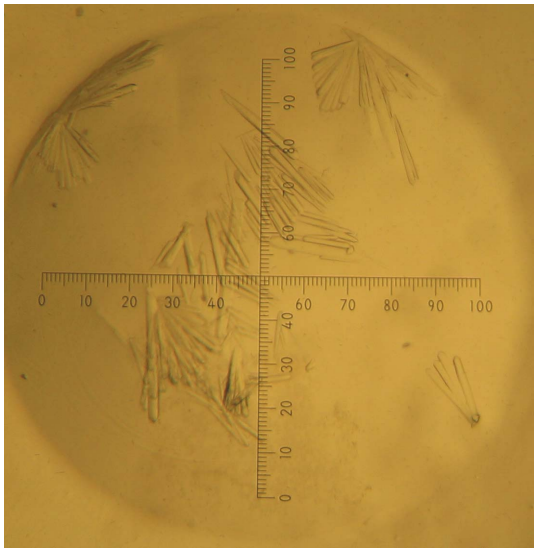

Figure 1
Crystals of the complex between rTxln-1 and trypsin grown from Hampton Crystal Screen 1 condition No. 27. These crystals are 0.3 × 0.03 × 0.01 mm in size. A larger crystal (see Table 1) was used for data collection.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

	rTxln-1–trypsin complex	Free rTxln-1
Unit-cell parameters (Å)	$a = b = 79.94$, $c = 107.83$	$a = b = 99.70$, $c = 77.47$
Space group	$P3_121$	$I422$
Temperature (K)	100	100
Resolution (Å)	42.60–2.00 (2.07–2.00)	38.55–1.63 (1.69–1.63)
Crystal dimensions (mm)	0.3 × 0.03 × 0.03	0.3 × 0.1 × 0.1
Mosaicity (°)	0.864	0.572
No. of observations	170492	224218
Unique reflections	27022	24804
Completeness	97.8 (99.8)	99.6 (98.8)
$\langle I/\sigma(I)\rangle$	13.7 (4.7)	16.9 (7.2)
$R_{\rm merge}$†	0.076 (0.345)	0.075 (0.360)

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h\rangle| / \sum_h \sum_i I_{h,i}$, where $I_{h,i}$ is the intensity of the ith measurement of reflection h and $\langle I_h\rangle$ is the average value over multiple measurements.

2.2. Preparation of the rTxln-1–trypsin complex

For crystallization trials, bovine trypsin (Sigma-Aldrich; Product No. T1426) and rTxln-1 were incubated for 30 min in the molar ratio 1:1.1. The mixture was purified by FPLC on a Superdex S200 column equilibrated with 25 m*M* Tris–HCl pH 7.4 and 50 m*M* NaCl. The fractions corresponding to the complex were pooled and assayed using the chromogenic substrate benzoyl-L-arginyl *p*-nitroanilide (BAPNA, Sigma-Aldrich). No significant residual activity could be measured. The sodium chloride was removed by buffer exchange and the complex concentrated to 10 mg ml^{-1} for crystallization trials. Free rTxln-1 was dialysed into 25 m*M* Tris–HCl pH 7.8 and concentrated to 40 mg ml^{-1} prior to crystallization trials.

2.3. Crystallization

Crystallization trials of the rTxln-1–bovine trypsin complex and free rTxln were carried out at 290 K by vapour-phase diffusion using hanging drops in VDX48 trays from Hampton. All of the wells had a volume of 200 µl, with the drops consisting of 1 µl well solution and 1 µl protein solution. Hampton Crystal Screens I and II and the Sigma Crystallization CryoKit (Sigma-Aldrich) were used in the trials. Sitting drops were also used in attempts to crystallize free rTxln-1. In these experiments, the concentration of rTxln-1 was allowed to vary between 5 and 40 mg ml^{-1}.

2.4. Cryocooling and data collection

Crystals of the rTxln-1–bovine trypsin complex were transferred from their growth drop to a new drop containing 70%(*v*/*v*) well solution and 30%(*v*/*v*) glycerol. These crystals were then soaked for

5 min in this solution before being looped directly into the cryostream. The free rTxln-1 crystals grew in solutions that did not require the further addition of cryoprotectant, thus allowing direct transfer to the cryostream.

X-rays were produced using a Molecular Structure Incorporated (MSI) FR-E X-ray generator operated at 45 kV and 45 mA. The images were recorded using an R-AXIS IV^{++} imaging-plate detector. For the complex, oscillation images were 0.5° in width, with exposure times of 240 s. For free rTxln-1, images were 1° in width, with exposure times of 300 s. During data collection, the crystal-to-detector distance was 130 mm for the crystals of the complex and 100 mm for the crystals of free Txln-1. Data for the complex were integrated, scaled and merged with the program *CrystalClear* 1.3.6 (Pflugrath, 1999), while *HKL*2000 was used for processing the free Txln-1 data (Otwinowski & Minor, 1997).

3. Results and discussion

Hampton Crystal Screen I condition Nos. 13, 38, 39 and 27 produced single crystals of the complex (Fig. 1), which diffracted to 5, 2.5, 2.3 and 2.0 Å resolution, respectively. Statistics for the data set to 2.0 Å resolution [condition No. 27; 0.1 *M* Na HEPES pH 7.5, 0.2 *M* trisodium citrate, 20%(*v*/*v*) 2-propanol] are presented in Table 1. Typically, three to four weeks of incubation were required to allow these crystals to grow to their maximum size. Crystals often grew in the presence of a significant amount of precipitate. In general, pH values in the range 7.5–8.5 and the presence of a low-molecular-weight organic compound were crucial factors in allowing crystallization to occur. All of the crystals examined belonged to space group $P3_121$ and had similar unit-cell parameters to the crystal from condition No. 7 of Hampton Screen 1 (Table 1). If it is assumed that there is one molecule in the asymmetric unit, V_M is 3.2 Å^3 Da^{-1} and the solvent content is 62%. Both values are within the range typical for most proteins (Matthews, 1968).

Initial attempts to crystallize free rTxln-1 at 5 mg ml^{-1} were unsuccessful, with virtually all of the trials yielding clear solutions. Subsequent trials at 15 mg ml^{-1} free rTxln-1 were also initially disappointing, with no crystals obtained in the first month of incubation. A survey of the literature showed that the presence of organic solvents such as phenol can promote crystallization of small disulfide-bonded peptides or small molecules. Porcine and human insulin (Whittingham *et al.*, 1999; Ciszak *et al.*, 1995) and α-dendrotoxin (Skarżyński, 1991) are examples where application of these additives was successful. Therefore, in the next round of trials phenol was added to a final concentration of 100–200 m*M* in both the drop and well solutions. This approach produced three conditions yielding X-ray diffraction-quality crystals. These were from Sigma CryoKit condition No. 13 [0.2 *M* sodium citrate, 0.1 *M* Tris–HCl pH 8.5, 30% PEG 400, 10%(*v*/*v*) glycerol], Hampton Crystal Screen 2 condition No. 13 [0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium sulfate, 30%(*w*/*v*) PEG monomethyl ether 2000], Hampton Crystal Screen 1 condition No. 9 [0.1 *M* trisodium citrate pH 5.6, 0.2 *M* ammonium acetate and 30%(*w*/*v*) PEG 4000] and Hampton Crystal Screen 1 condition No. 39 [0.1 *M* HEPES pH 7.5, 2.0 *M* ammonium sulfate, 2%(*v*/*v*) PEG 400]. These crystals generally diffracted to a resolution between 3.0 and 2.7 Å. Two other conditions, Hampton Crystal Screen 2 condition No. 14 (0.1 *M* trisodium citrate pH 5.6, 2.0 *M* ammonium sulfate and 0.2 *M* potassium sodium tartrate) and Sigma CryoKit condition No. 13 [0.1 *M* Tris–HCl pH 8.5, 0.2 *M* sodium citrate, 30%(*v*/*v*) PEG 400] with 100 m*M* phenol added also yielded small needle-shaped crystals, but their diffraction patterns were not analysed.

After six months incubation in the absence of adding any phenol, large single crystals appeared in the original drops that were set up with 15 mg ml^{-1} free rTxln-1. The best of these crystals were obtained from Hampton Crystal Screen I condition No. 39 [0.1 *M* Na HEPES pH 7.5, 2%(*v*/*v*) PEG 400 and 2 *M* ammonium sulfate]. These crystals diffracted X-rays to 1.63 Å resolution (Fig. 2) and belonged to space group *I*422 (Table 1). The space group and unit-cell para-

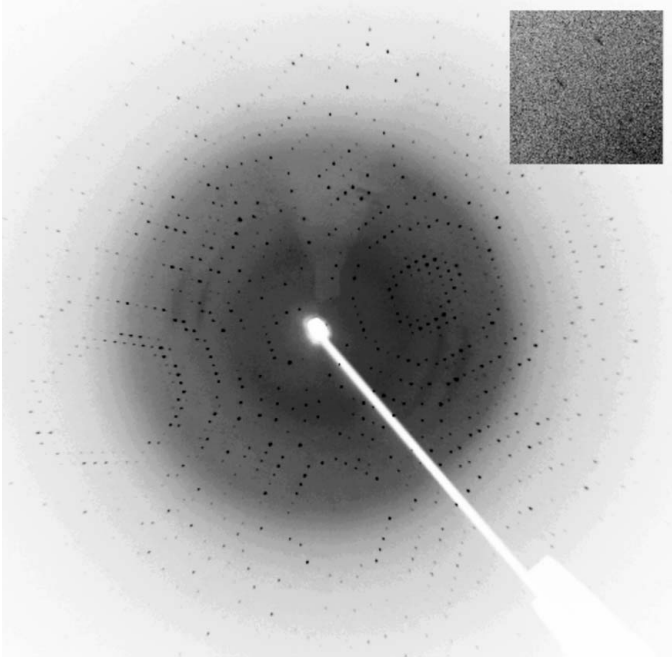

Figure 2
A 1° oscillation frame of a cryocooled crystal of rTxln-1. Diffraction data were observed to 1.63 Å resolution. The inset is centred on the 1.7 Å resolution ring.

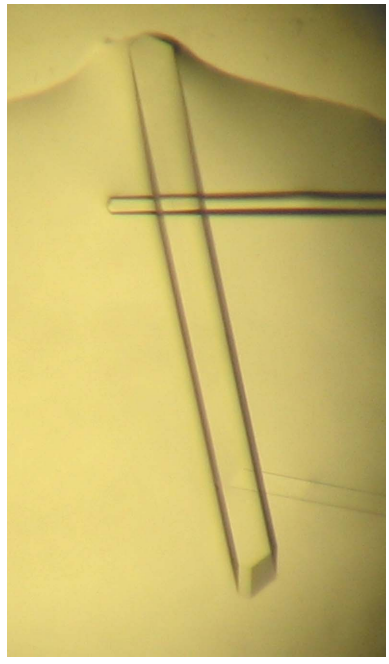

Figure 3
Crystals of free rTxln-1 grown from Hampton Crystal screen 2 condition No. 14 with 1,4-butanediol as an additive. The largest crystal in the drop is 0.6 × 0.1 × 0.1 mm in size.

meters were common to all of the crystals of free rTxln-1 that were tested by X-ray analysis. Similar crystals that diffracted to this resolution were also grown when 10%(*v*/*v*) 1,4-butanediol was added to condition No. 39 (Fig. 3). In other subsequent experiments we have also demonstrated that the rate of crystallization can be enhanced significantly by increasing the concentration of free rTxln-1 up to 40 mg ml^{-1}.

Based on a V_M of 2.4 Å^3 Da^{-1} and a solvent content of ∼49% (Matthews, 1968), it is likely that there are three molecules of free rTxln-1 in the asymmetric unit. We are now attempting to determine the three-dimensional structures of these molecules and crystallize rTxln-1 in complex with plasmin, its putative envenomation target.

We thank Dr Stan Bastiras (BresaGen Ltd) for producing the recombinant textilinin-1 for this work. We acknowledge the kind support of Professor Patrick Gaffney, who originally highlighted the unique kinetics of textilinin-1 and the naming of the molecule. This work was supported by an ARC-Linkage grant LP0454264 funded by the Australian Research Council and by QRxPharma. We thank Karl Byriel for assistance with data collection.

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