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Crystallization and preliminary X-ray diffraction data analysis of stenodactylin, a highly toxic type 2 ribosome-inactivating protein from *Adenia stenodactyla*

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Ribosome-inactivating proteins (RIPs) inhibit protein synthesis and induce cell death by removing a single adenine from a specific rRNA loop. They can be divided into two main groups: type 1 and type 2 RIPs. Type 1 RIPs are single-chain enzymes with N-glycosidase activity. Type 2 RIPs contain two chains (A and B) linked by a disulfide bond. The A chain has RIP enzymatic activity, whereas the B chain shows lectin activity and is able to bind to glycosylated receptors on the cell surface. Stenodactylin is a type 2 RIP from the caudex of *Adenia stenodactyla* from the Passifloraceae family that has been recently purified and characterized. It shows a strong enzymatic activity towards several substrates and is more cytotoxic than other toxins of the same type. Here, the crystallization and preliminary X-ray diffraction data analysis of stenodactylin are reported. This RIP forms crystals that diffract to high resolution (up to 2.15 Å). The best data set was obtained by merging data collected from two crystals. Stenodactylin crystals belonged to the centred monoclinic space group *C*2 and contained two molecules in the asymmetric unit.

1. Introduction

Ribosome-inactivating proteins (RIPs) have a broad distribution in nature, especially in the plant kingdom (Girbes *et al.*, 2004). They are specific RNA N-glycosidases (EC 3.2.2.22) that depurinate a conserved adenine in an exposed loop of rRNA in the largest subunit of the ribosome (Endo & Tsurugi, 1987). RIPs have been classified into two main groups, type 1 and type 2 RIPs, with the first group being larger than the second. Type 1 RIPs are strongly basic single-chain enzymes with a molecular weight of about 30 kDa. Type 2 RIPs, or toxic lectins, are proteins with a molecular weight of about 60–65 kDa that consist of an A chain with the same enzymatic activity as a type 1 RIP and a B chain linked together by a disulfide bond. The B chain is a lectin specific for galactose, *N*-acetyl galactosamine or *N*-acetyl neuraminic acid (van Damme *et al.*, 2001). The enzymatic activity of RIPs causes protein-synthesis arrest, targeting the rRNA loop involved in elongation-factor binding. All RIPs also remove adenine from DNA and other polynucleotides; the name 'polynucleotide adenine glycosylases' has therefore been proposed (Barbieri *et al.*, 1997, 2001).

The toxicity of RIPs to cells and consequently to animals is highly variable and depends on their structure: in general, type 1 RIPs have a lower toxicity than type 2 RIPs (Stirpe & Battelli, 2006). The B chain facilitates the entry of the molecule into the cell by binding to sugars on the cell membrane, whereupon the A chain can damage ribosomes and possibly other structures, finally causing cell death.

Several type 2 RIPs are potent toxins, some of which, such as ricin, have been known for a long time (Olsnes, 2004). RIPs have been used for criminal and even warfare or bioterrorism purposes (Bigalke & Rummel, 2005; Audi *et al.*, 2005). However, they have also found medical applications in cancer therapy when converted into very potent and specific drugs by conjugation with antibodies or other molecules that act as site-directing carriers (Bolognesi & Polito, 2004; Pastan *et al.*, 2007).

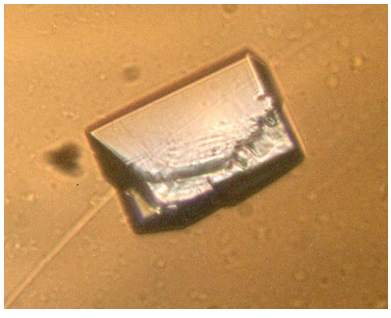

Table 1
Physical chemical and biological characteristics of stenodactylin (Pelosi *et al.*, 2005; Stirpe *et al.*, 2007).

Molecular mass from SDS–PAGE (Da)	
Whole molecule	53800
A chain	24800
B chain	30000
Molecular mass from HPLC/MS (Da)	63131
Isoelectric point (pH)	4.8–5.0
Carbohydrate side chains	Present
Haemagglutinating activity† (*M*)	7.9×10^{-7}
Inhibitory activity on protein synthesis‡ (μg ml^{-1})	
Unreduced	5.6
Reduced	0.5
Adenine glycosylase activity§ (pmol)	4.55

† Minimum agglutinating concentration. ‡ Concentration giving 50% inhibition in a rabbit reticulocyte lysate system (IC_{50}). § Towards rat liver ribosomes (picomoles of adenine released per 10 pmol lectin in 40 min).

Despite the similarities in their structures, type 2 RIPs show differences in the lesions that they cause in experimental animals, including their effects on the nervous system. All the toxic lectins tested, namely ricin, abrin, modeccin and viscumin, are retrogradely transported when injected into peripheral nerves, whereas only modeccin, volkensin and stenodactylin are retrogradely transported when injected into the central nervous system (Wiley & Kline, 2000; Monti *et al.*, 2007).

Stenodactylin is a recently characterized type 2 RIP (Stirpe *et al.*, 2007). It was purified from the caudex of *Adenia stenodactyla*, a plant belonging to the Passifloraceae family (Pelosi *et al.*, 2005). Stenodactylin has a high enzymatic activity towards ribosomes and hsDNA substrates and is probably the most cytotoxic type 2 RIP described to date (Stirpe *et al.*, 2007). In the rat central nervous system, stenodactylin is taken up and retrogradely transported from the terminal field of nerve cells to the cell bodies. Its toxic action results in a significant depletion of medial septum cholinergic neurons that project into the injected area of the hippocampus (Monti *et al.*, 2007). Stenodactylin is highly toxic to mice and also shows a strong systemic toxicity, causing death in 100% of mice injected with 1.21 μg kg^{-1} within 7 d (Stirpe *et al.*, 2007). This activity is comparable to or even higher than that observed with volkensin, the most toxic lectin of plant origin known to date (Stirpe *et al.*, 1985).

The astonishingly high biological activity of stenodactylin makes the study of its structure–function relationship exceedingly important. In this paper, crystallization conditions and preliminary X-ray diffraction data analysis of stenodactylin are reported.

2. Protein purification

Stenodactylin was extracted and purified from the caudices of *A. stenodactyla* as described in Pelosi *et al.* (2005). Briefly, the protein was precipitated in 100% saturated ammonium sulfate and the precipitate was dissolved in and dialyzed against PBS pH 7.5. The protein was loaded onto an acid-treated Sepharose CL-6B column and eluted as a single peak with 0.2 *M* galactose. The main physical chemical and biological characteristics of stenodactylin are reported in Table 1 (Pelosi *et al.*, 2005; Stirpe *et al.*, 2007).

3. Protein crystallization

Stenodactylin was crystallized by the conventional sitting-drop vapour-diffusion technique in a Linbro multi-well crystallization plate at 293 K. The droplets (4 μl) contained equal volumes of protein solution and reservoir solution. 750 μl reservoir solution was poured into each well. The best condition for the growth of single crystals consisted of 1.4–1.5 *M* sodium malonate pH 6.8–7.5 as the reservoir and a protein concentration of 5.76 mg ml^{-1} in 5 m*M* sodium phosphate buffer pH 7.0, 0.14 *M* NaCl and 4 m*M* galactose.

Crystals initially appeared after three weeks and grew in about one month to maximum dimensions of approximately 0.3 × 0.2 × 0.1 mm (Fig. 1).

4. Data collection, processing and preliminary X-ray analysis

Several crystals were tested in X-ray diffraction experiments. Each crystal was first briefly soaked in a cryoprotectant solution containing 2.0 *M* sodium malonate at the same pH as the reservoir solution (6.8–7.5) and 20%(*v*/*v*) glycerol and then transferred into a cold N_2-gas stream (100 K) for data collection. Data were collected using an

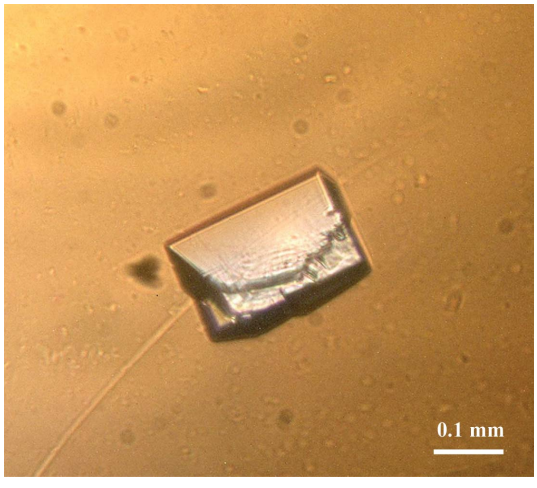

Figure 1
Optical micrograph of a crystal of stenodactylin.

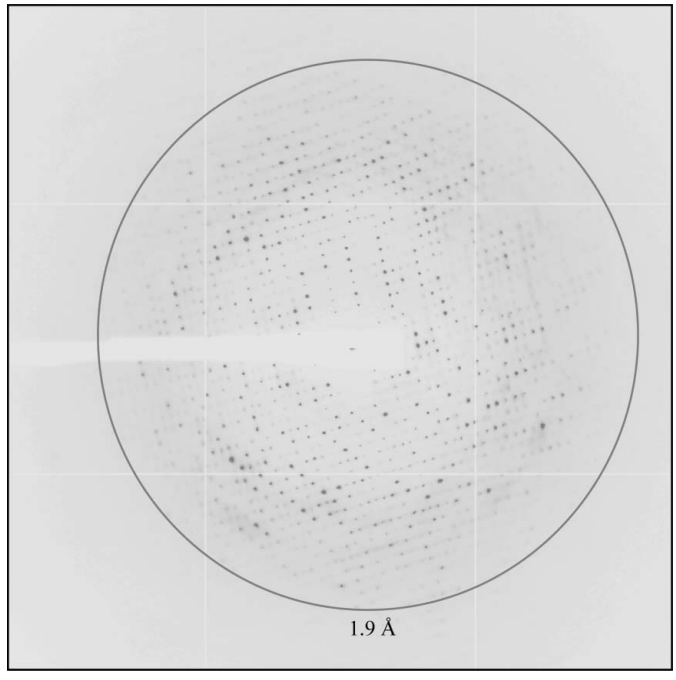

Figure 2
X-ray diffraction pattern from a crystal of stenodactylin.

Table 2
X-ray data-collection statistics for stenodactylin.

Values in parentheses are for the last resolution shell.

Unit-cell parameters (Å, °)	a = 220.54, b = 64.26, c = 91.75, β = 105.68
Space group	$C2$
Resolution (Å)	50.0–2.15 (2.23–2.15)
Observed reflections	612.719
Unique reflections	64.284
Mosaicity (°)	0.885
Completeness (%)	94.0 (72.9)
$\langle I/\sigma(I)\rangle$	18.7 (2.5)
Redundancy	9.5 (7.0)
$R_{\rm merge}$†	0.183 (0.574)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the measurement of an equivalent reflection with indices *hkl*.

ADSC Quantum CCD detector on beamlines ID29 and ID14-1 at the ESRF (Grenoble, France) using wavelengths of 0.976 and 0.934 Å and crystal-to-detector distances of 283.88 and 265.31 mm, respectively. In both experiments a $\Delta\varphi$ oscillation of 1° was used. A typical diffraction pattern is shown in Fig. 2. The best crystal diffracted to a resolution of 1.90 Å, but the data were trimmed to 2.15 Å to achieve good values of completeness and average $I/\sigma(I)$ in the last resolution shell. The data were processed and scaled using the *DENZO* and *SCALEPACK* programs (Otwinowski & Minor, 1997). All the tested crystals diffracted in accordance with a centred monoclinic space group with similar unit-cell parameters. The unit-cell parameters and data-collection statistics obtained by merging two data sets collected from two crystals are reported in Table 2. Further resolution cuts up to 2.5 Å did not significantly improve the $R_{\rm merge}$ [$R_{\rm merge}$ and $\langle I/\sigma(I)\rangle$ are 0.017 and 40.0, respectively, at 2.5 Å, and are 0.435 and 4.3 in the last resolution shell].

Matthews coefficient calculations (Matthews, 1968) indicated the presence of two heterodimers in the asymmetric unit, corresponding to a $V_{\rm M}$ of 2.61 Å^3 Da^{-1} and a solvent content of 52.5%. The amino-acid sequence of stenodactylin is only partially known: about 20 amino acids in the N-terminal regions of the A and B chains have been sequenced. Amino-acid sequence alignment of these short regions with those of other type 2 RIPs indicates a high homology with the A and B chains of volkensin, the structure of which is unknown. A molecular model of volkensin has been elaborated on the basis of the crystallographic coordinates of ricin (Chambery *et al.*, 2004), which shares a high sequence homology with volkensin.

Molecular-replacement procedures using the program *EPMR* (Kissinger *et al.*, 1999) with the structure of ricin as a probe (PDB code 2aai; Rutenber *et al.*, 1991) clearly showed two solutions. The correlation coefficient (CC) and R factor corresponding to the best solutions were CC = 0.134, R = 0.593 for the first solution and CC = 0.322 and R = 0.628 for the second solution. Refinement cycles of the structure and amino-acid sequencing are in progress.

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