Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction analysis of two ribosome-inactivating proteins: lychnin and dianthin 30

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Received 16 April 2003
Accepted 13 May 2003

Lychnin from the seeds of *Lychnis chalcedonica* and dianthin 30 from the leaves of *Dianthus caryophyllus* belong to the type 1 ribosome-inactivating proteins (RIPs). They have been crystallized by the vapour-diffusion method and the crystals diffracted to 1.7 and 1.3 Å, respectively, using a synchrotron source. Lychnin and dianthin 30 crystals both belong to space group $P2_1$ with one protein chain in the asymmetric unit. The structure of dianthin 30 has been solved by molecular replacement using the coordinates of saporin-S6 as a model. The structure determination of lychnin, the sequence of which is not yet available, is in progress using the coordinates of other RIPs as models for molecular replacement.

1. Introduction

Ribosome-inactivating proteins (RIPs) are produced by a wide variety of plants (Barbieri *et al.*, 1993; Van Damme *et al.*, 2001). It has been shown (Endo *et al.*, 1987; Endo & Tsurugi, 1987) that the enzymatic activity of RIPs consists of a specific cleavage of the N-glycosidic bond between the ribose and adenine corresponding, for example, to residue A_{4324} in rat liver ribosome 28S rRNA (rRNA N-glycosidase activity). This adenine belongs to the α-sarcin loop, which is conserved in large rRNAs from bacteria, plants and animals. This irreversible modification causes a loss of interaction between the ribosomes and the elongation factors 1 and 2, with consequent inhibition of protein synthesis. It was subsequently found that all RIPs also remove adenine from DNA and other polynucleotides (Barbieri *et al.*, 1997), hence the denomination 'polynucleotide adenine glycosylases' proposed for these proteins (Barbieri *et al.*, 2001).

RIPs are classified into three groups based on their physical properties and structure (Mundy *et al.*, 1994). Type 1 RIPs are monomeric enzymes with an approximate molecular weight of 30 kDa. They are usually very basic, with pI values around or above 10. Type 2 RIPs, such as ricin, are heterodimeric proteins. The *A* chain (catalytic subunit), which has the same enzymatic properties as a type 1 RIP, is linked to the *B* chain, which is a galactose-binding lectin (MW $\simeq$ 30 kDa), through a disulfide bond. The *B* chain binds to galactose residues on the cell surface, thus allowing the entry of the *A* chain into cells, where it exerts its enzymatic activity. Thus, some, but not all, type 2 RIPs are potent toxins (ricin and related toxins), whereas type 1 RIPs can only enter into cells with difficulty and consequently have low cytotoxicity, although they are very active towards isolated ribosomes. The term 'type 3 RIP' was introduced by Mehta & Boston (1998) for maize RIP b-32, which is composed of two polypeptides of 16.5 and 8.5 kDa.

However, Van Damme *et al.* (2001) propose considering this as a 'two-chain type 1 RIP' and to limit the denomination 'type 3 RIP' to JIP60 maize RIP type 3. The latter consists of an N-terminal domain similar to other type 1 RIPs linked to an unrelated C-terminal domain of unknown function (Reinbothe *et al.*, 1994).

Although the biological role of RIPs seems to be very complex, their function has been related to defence against viral or fungal infections in plants (Leeds *et al.*, 1991). RIPs are important for their applications in agriculture, as plants transfected with RIP genes show an increased resistance to viruses. In medicine, their use in therapy for HIV infection has been proposed and complexes constituted of monomeric RIPs and monoclonal antibodies (immunotoxins) have been prepared to be directed specifically against malignant and other harmful cells (Pastan & Fitzgerald, 1991; Stirpe *et al.*, 1992). The potential use of RIPs as cell-destructive agents has stimulated efforts to isolate and characterize such proteins from many different plant sources. Although RIPs show similar physicochemical properties and identical enzymatic activities (Stirpe *et al.*, 1986), they act differently on ribosomes from various plants (Stirpe *et al.*, 1986), protozoa (Cenini *et al.*, 1988) and animals (Brigotti *et al.*, 1989); for this reason, they could be useful tools in the study of ribosomal properties.

Lychnin (MW $\simeq$ 26 kDa) and dianthin 30 (MW = 32.7 kDa) belong to the type 1 RIPs. They have been extracted and purified from

the seeds of *Lychnis chalcedonica* (Bolognesi *et al.*, 1990) and from the leaves of *Dianthus caryophyllus* (Stirpe *et al.*, 1981), respectively.

Here, we report the crystallization conditions and the preliminary X-ray analysis of both lychnin and dianthin 30.

2. Materials and methods

2.1. Purification and crystallization

Lychnin and dianthin 30 were purified as previously described by Bolognesi *et al.* (1990) and by Stirpe *et al.* (1981), respectively. Purified and lyophylized lychnin was dissolved to a concentration of 10 mg ml^{-1} in a solution containing 5 m*M* sodium phosphate pH 7.5 and 0.14 *M* NaCl. Initial crystallization trials were carried out by the sitting-drop vapour-diffusion method using the JBScreen system (Jena Bioscience). Each drop was prepared by mixing equal volumes (2.5 µl) of protein solution and reservoir and was equilibrated against 700 µl of the reservoir at 293 K. Crystals were obtained using solution C3 of JBScreen 5. The condition was further optimized by variation of pH and ionic strength. The best crystals (Fig. 1) grew in few days using a reservoir solution containing 30%(*w*/*v*) PEG 8000 and 0.1 *M* sodium phosphate pH 6.5–7.2.

Dianthin 30 (16.9 mg ml^{-1} in 5 m*M* sodium acetate pH 4.5) was crystallized by the hanging-drop vapour-diffusion technique at 293 K. 2.5 µl droplets of protein solution were mixed with an equal volume of reservoir solution and equilibrated against 750 µl of the same solution. Crystals with various morphologies were obtained from several conditions of Crystal Screens I and II from Hampton Research (Jancarik & Kim, 1991): condition 4 from Crystal Screen I (2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5) (Fig. 2*a*), condition 10 from Crystal Screen I [0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 4.5, 30%(*w*/*v*) PEG 4000] (Fig. 2*b*), condition 20 from Crystal Screen II (1.6 *M* magnesium sulfate, 0.1 *M* MES pH 6.5) (Fig. 2*c*), condition 31 from Crystal Screen I [0.2 *M* ammonium sulfate, 30%(*w*/*v*) PEG 4000] (Fig. 2*d*), condition 39 Crystal Screen I [2%(*v*/*v*) PEG 400, 2.0 *M* ammonium sulfate, 0.1 *M* HEPES pH 7.5] (Fig. 2*e*) and condition 5 from Crystal Screen II [2 *M* ammonium sulfate, 5%(*v*/*v*) 2-propanol] (Fig. 2*f*).

2.2. Data collection

Data were collected from lychnin crystals using synchrotron radiation (Elettra X-ray diffraction beamline, Trieste, Italy) at 100 K. The crystals were soaked for few seconds in a cryogenic solution containing 30%(*w*/*v*) PEG 8000 and 30%(*v*/*v*) PEG 400, and rapidly exposed to a cold nitrogen stream (Oxford Cryosystems Cryostream). The diffraction data were collected from a single crystal (of dimensions 400 × 50 × 50 µm) to a resolution of 1.7 Å (Fig. 3*a*) with a MAR Research CCD using a radiation wavelength of 1.0 Å, an oscillation angle of 2.0° and a crystal-to-detector distance of 120 mm.

The diffraction data for dianthin 30 were collected from a single crystal (of dimensions 360 × 150 × 90 µm) grown using condition 10 of Crystal Screen I (Fig. 2*b*) at ESRF, Grenoble, France (beamline ID29) at 100 K. After a brief soak in a cryogenic solution [33%(*w*/*v*) PEG 4000 and 20%(*v*/*v*) PEG 400], the crystal diffracted to 1.3 Å resolution (Fig. 3*b*). The data were recorded on a CCD ADSC Quantum-210 detector using a wavelength of 1 Å, an oscillation angle of 2.0° and a crystal-to-detector distance of 120 mm.

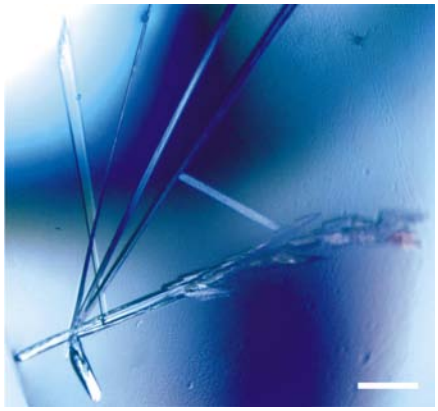

Figure 1
Crystals of lychnin grown by the sitting-drop vapour-diffusion method. The scale bar is 0.2 mm.

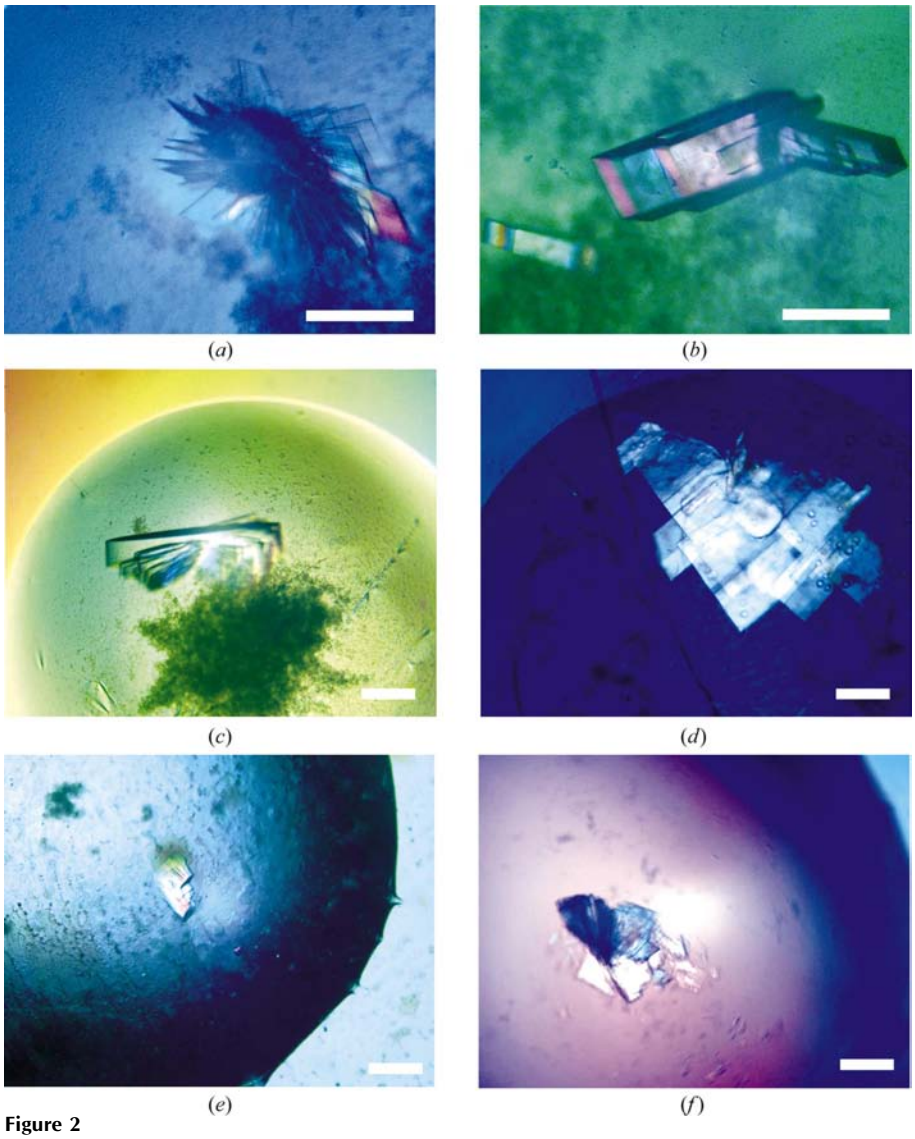

Figure 2
Crystals of dianthin 30 grown as described in §2.1. Scale bars are 0.3 mm.

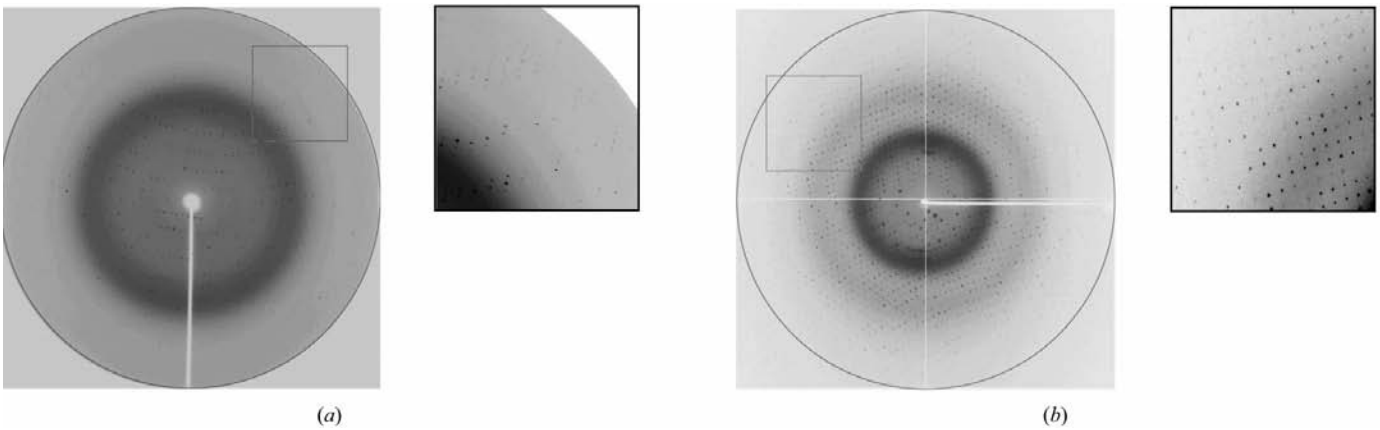

Figure 3
X-ray diffraction patterns for (*a*) lychnin and (*b*) dianthin 30.

Table 1
Unit-cell parameters and data-collection statistics for lychnin and dianthin 30.

Values in parentheses refer to the last resolution shell: 1.76–1.70 Å for lychnin and 1.45–1.40 Å for dianthin 30.

	Lychnin	Dianthin 30
No. of crystals used	1	1
Data-collection time (h)	3	1
Rotation range (°)	360	360
Unit-cell parameters (Å, °)	a = 35.25, b = 57.33, c = 52.23, β = 106.3	a = 37.57, b = 78.65, c = 43.14, β = 105.4
Space group	$P2_1$	$P2_1$
Resolution limits (Å)	29.1–1.7	39.2–1.4
Mosaicity (°)	0.7	0.4
Measured reflections	151602	295523
Unique reflections	21879	44521
Rejected reflections	850	1802
Completeness (%)	99.4 (98.3)	94.0 (90.6)
Redundancy	6.9	6.6
$I/\sigma(I)$	39.7 (14.2)	26.3 (10.4)
R_{sym}	0.048 (0.084)	0.063 (0.255)

3. Results and discussion

The diffraction data were processed and scaled for both proteins with the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997). The data reduction, including a search for systematic absences, clearly indicates that lychnin and dianthin 30 crystals belong to space group $P2_1$. The unit-cell parameters and the data-collection statistics are listed in Table 1.

Matthews coefficient (Matthews, 1968) calculations show the presence of one chain in the asymmetric unit in both crystals. The V_M values are 1.95 Å^3 Da^{-1} for lychnin and 1.92 Å^3 Da^{-1} for dianthin 30, with solvent contents of 36.4 and 35.4%, respectively.

Type 1 RIPs exhibit high sequence similarity to each other; in particular, dianthin 30 shows 87% homology with saporin-S6 from *Saponaria officinalis*. Therefore, its structure (Savino *et al.*, 2000; PDB code 1qi7) was used as a search model for the molecular-replacement procedure. The best solution has a correlation coefficient (CC) of 0.470 and an R factor of 0.407, while for the second-best solution the CC is 0.129 and the R factor is 0.526.

The crystal packing built from the coordinates of the best solution was checked. A Fourier map calculated after rigid-body refinement showed the electron density of most of the molecule. Further refinement for dianthin 30 is now in progress.

Although the sequence of lychnin is not known, molecular-replacement calculations were performed using various models such as ricin chain *A* (Weston *et al.*, 1994; PDB code 1ift), PAP (Monzingo *et al.*, 1993; PDB code 1paf) and saporin-S6 (Savino *et al.*, 2000; PDB code 1qi7). The most promising solution has been obtained with PAP as a probe, but further attempts with other models are in progress.

Financial supports from University of Bologna (funds for selected research topics), from Ministero della Salute and from Pallotti's Legacy for Cancer Research are gratefully acknowledged. We thank ESRF (Grenoble) and Elettra (Trieste) for providing access to crystallographic experimental facilities.

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