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Crystallization and preliminary X-ray crystallographic studies on a Kunitz-type potato serine protease inhibitor

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Interest in protease inhibitors has been renewed because of their potent activity in preventing carcinogenesis in a wide variety of *in vivo* and *in vitro* model systems. Potato tubers contain a wide range of such protease inhibitors. In cv. *Elkana* potato tubers, protease inhibitors represent about 50% of the total amount of soluble protein. Potato serine protease inhibitor (PSPI), one of the isoforms of the most abundant group of protease inhibitors, is a dimeric double-headed Kunitz-type inhibitor. No high-resolution structural information on this type of inhibitor has so far been obtained, as all currently known structures are of the monomeric single-headed or monomeric double-headed types. Crystals were grown in 0.1 *M* HEPES pH 7.5, 10% PEG 8000 and 8% ethylene glycol complemented with 9 m*M* 1-*s*-octyl-β-D-thioglucoside or 0.1 *M* glycine. Data were collected from a single crystal under cryoconditions to 1.8 Å resolution. The protein crystallized in space group $P2_1$, with unit-cell parameters a = 54.82, b = 93.92, c = 55.44 Å, β = 100.7°; the scaling $R_{\rm sym}$ is 0.044 for 45 456 unique reflections.

1. Introduction

The majority of known and characterized protease inhibitors are serine protease inhibitors and have been classified into at least 13 different families according to their sequence homology (Richardson, 1991). Protease inhibitors are denoted as Kunitz-type inhibitors when particular conserved amino acids are present in the primary structure, such as four cysteinyl residues forming two disulfide bridges (Richardson, 1991). The Kunitz-type inhibitors are relatively small (approximately 20 kDa) and are mostly monomeric and single-headed, containing one reactive site that inhibits the protease. Kunitz-type inhibitors are pseudo-substrates that bind directly to the binding site of the protease. To date, only monomeric single-headed (*e.g.* winged-bean chymotrypsin inhibitor) or monomeric double-headed inhibitors (*e.g.* soybean trypsin inhibitor) have been crystallized. PSPI is a dimeric double-headed Kunitz-type inhibitor with two subunits that differ in size (15 kDa for the larger subunit and 6 kDa for the smaller subunit; Pouvreau *et al.*, 2003). The subunits share about 22% sequence homology with soybean trypsin inhibitor, the reference inhibitor for the Kunitz type (Kunitz, 1945). Here, we describe the crystallization conditions of a dimeric Kunitz-type inhibitor which inhibits trypsin, chymotrypsin and human leukocyte elastase (Pouvreau *et al.*, 2001).

2. Materials and methods

2.1. Purification

Potatoes of cv. *Elkana* (Avebe BA, Veendam, The Netherlands) were stored at 277 K in the dark at a relative humidity of 95–100% for a maximum period of six months. The potatoes were chopped into large pieces and mixed in the presence of sodium bisulfite (0.5 g per kilogram of potatoes) to prevent the oxidation of phenolic compounds. Potato juice was prepared as described previously (Pouvreau *et al.*, 2001). PSPI 6.1 (the number representing the isoelectric point), one of the isoforms of the PSPI group, was purified as described previously (Pouvreau *et al.*, 2001). PSPI 6.1 is a dimeric protein of molecular weight 20 273 Da (Pouvreau *et al.*, 2003) in which the two subunits are disulfide-linked; it shows approximately 22% sequence homology with the Kunitz soybean trypsin inhibitor. An additional chromatofocusing step was included using a Polybuffer Exchanger 74 column (60 × 1.6 cm). The fraction containing PSPI 6.1 was loaded onto the column, which had been pre-equilibrated with 0.025 *M* imidazole–HCl buffer pH 7.4, and the protein was eluted using Polybuffer 74–HCl pH 5.0 (dilution factor 1:8; Amersham Biosciences, Uppsala, Sweden). The Polybuffer was removed using a HP Phenyl Sepharose column (10 × 2.6 cm). The chromatofocusing step resulted in the removal of minor contaminants. After purification,

PSPI 6.1 was dialyzed at 277 K against 10 m*M* Tris–HCl buffer pH 8.0 and subsequently stored (253 K) until use at a concentration of 1 mg ml^{-1}.

2.2. Crystallization

The purified PSPI 6.1 was concentrated to 10 mg ml^{-1} in 10 m*M* Tris–HCl buffer pH 8.0 using a Centripep 10 kDa ultracentrifugation device (Millipore, Bedford, MA, USA).

Crystallization experiments were performed by the sitting-drop vapour-diffusion method at 295 K. Each drop was formed by mixing equal volumes (1 µl) of 10 mg ml^{-1} PSPI 6.1 solution and reservoir solution. Crystals were grown (Fig. 1) in 0.1 *M* HEPES pH 7.5, 10% PEG 8000 and 8% ethylene glycol complemented with 9 m*M* 1-*s*-octyl-β-D-thioglucoside (Hampton Detergent Screen 1) or 0.1 *M* glycine (Hampton Additive Screen 2).

(*a*)

(*b*)

Figure 1
Crystals of potato serine protease inhibitor (PSPI 6.1) from 0.1 *M* HEPES pH 7.5, 10% PEG 8000 and 8% ethylene glycol with the following detergents or additives: (*a*) 0.1 *M* glycine, (*b*) 9 m*M* 1-*s*-octyl-β-D-thioglucoside.

2.3. X-ray data collection and processing

A single crystal was soaked in reservoir solution containing 25% glycerol for 10 s and mounted on a nylon-fibre loop (Hampton), after which it was flash-frozen in a liquid-nitrogen stream at 100 K. The data were collected at beamline ID29 of the European Synchrotron Radiation Facility (ESRF) on an ADSC Q4R detector. The crystal-to-detector distance was 200 mm and 100° of 1° oscillation images (three passes of 0.5 s per frame) were collected (λ = 0.9464 Å). The intensities were indexed with *MOSFLM* (Leslie, 1999) and scaled using *SCALA* (Evans, 1993). Molecular replacement was performed using *AMoRe* (Navaza, 1994), *MOLREP* (Vagin & Teplyakov, 1997) and *BEAST* (Read, 2001), which are all available in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

3. Results

PSPI 6.1 was purified and crystallized as shown in Fig. 1. A data set was collected from a single crystal to 1.8 Å; because of the use of a square detector, the completeness in the highest resolution shell (1.90–1.80 Å) is only 52.1%. In Fig. 2, an X-ray diffraction pattern of a PSPI 6.1 crystal is shown. The data was processed in space group $P2_1$, with unit-cell parameters a = 54.82, b = 93.92, c = 55.44 Å, β = 100.7°. See Table 1 for data-collection and processing parameters. The exact Matthews coefficient (V_M) and solvent content of the crystals could not be determined. Neither a self-rotation function nor a native Patterson indicated a clear rotational or translational symmetry between molecules in the asymmetric unit (AU). If there are two molecules in the AU, V_M = 3.5 Å^3 Da^{-1}, corresponding to a solvent content of 64.2%, whilst three molecules in the AU gives a V_M of 2.3 Å^3 Da^{-1} and a solvent content of 46.2%. According to the Matthews coefficient probabilities (Kantardjieff & Rupp, 2003), the latter seems the more likely. We were not able to solve the structure by molecular replacement using the winged-bean chymotrypsin inhibitor (Ravichandran *et al.*, 1999), which has 32% sequence homology, or the Kunitz-type soybean trypsin inhibitor as a model. Structure solution by heavy-atom methods is in progress.

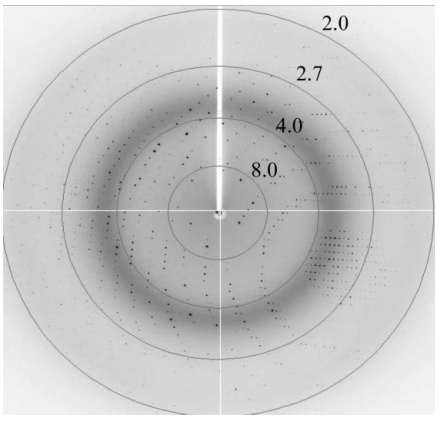

Figure 2
X-ray diffraction pattern of a potato serine protease inhibitor (PSPI 6.1) crystal. The resolutions are given in Å.

Table 1
Data-collection and processing parameters.

Data statistics for the outer resolution shell (1.90–1.80 Å) are given in parentheses where applicable.

Crystal dimensions (mm)	0.4 × 0.025 × 0.025
Temperature (K)	295
Wavelength (Å)	0.94644
Resolution range (Å)	54.23–1.80
Crystal system	Monoclinic
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 54.82, b = 93.92, c = 55.44, β = 100.7
Total No. reflections	89852
No. unique reflections	45456
R_{sym}†	0.044 (0.13)
Completeness (%)	88.7 (52.1)‡
Average $I/\sigma(I)$	4.4 (5.6)

† $R_{sym} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i \langle I_h \rangle$, where I_{hi} is the intensity of the ith measurement of the same reflection and $\langle I_h \rangle$ is the mean observed intensity for that reflection. ‡ Data in the highest resolution shell are less complete owing to data collection on a square detector.

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