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Crystallization, data collection and phasing of black-eyed pea trypsin/chymotrypsin inhibitor in complex with bovine β-trypsin

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The black-eyed pea trypsin and chymotrypsin inhibitor (BTCI) is a Bowman–Birk-type inhibitor from *Vigna unguiculata* seeds. A complex of BTCI with bovine β-trypsin was crystallized by the hanging-drop vapour-diffusion method with ammonium sulfate as precipitant. Crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 59.3, b = 61.8, c = 80.0 Å. Diffraction data were collected to 2.36 Å resolution and were processed to give an overall $R_{\rm sym}$ of 0.137. The Matthews coefficient for one complex per asymmetric unit is 2.2 Å^3 Da^{-1}, with a corresponding solvent content of 43%. After molecular replacement and initial refinement, the model gives an $R_{\rm cryst}$ of 0.361 and an $R_{\rm free}$ of 0.432.

1. Introduction

Serine proteases are enzymes that cleave peptide bonds of proteins within a specific sequence. These enzymes have diverse biological functionalities that may be harmful to other organisms. In biological systems, proteases are controlled by several mechanisms, including inactivation by proteolytic degradation or by interaction with inhibitors, which act as pseudo-substrates displaying variable degrees of affinity for the enzyme's catalytic sites (Laskowski & Kato, 1980; Bode & Huber, 2000). Natural inhibitors of these enzymes have been well characterized and their structural features have been explored as a tool in the investigation of the catalytic mechanism of serine proteases (Blow *et al.*, 1974; Bode & Huber, 2000). Most of these inhibitors bind to cognate enzymes according to a common substrate-like standard mechanism (Grutter *et al.*, 1990).

Serine protease inhibitors have been classified into 18 families on the basis of their primary structure and the disulfide bond (Bode & Huber, 1992; Laskowski & Quasim, 2000). Two main families were extensively investigated and characterized from a structural point of view: the Kunitz family, whose members have a molecular mass of about 20 kDa and usually have two disulfide bonds, and the Bowman–Birk family, whose members have a molecular mass of about 8–15 kDa and have seven disulfide bonds.

Interest in understanding the physiological roles of protease inhibitors has increased owing to their regulatory action in different processes involving proteases, such as intracellular protein breakdown, transcription, the cell cycle, cell invasion and apoptosis (Fumagalli *et al.*, 1996; Thompson & Palmer, 1998; Kato, 1999), as well as being important factors in response to abiotic (Franco & Melo, 2000) and biotic stress (Gatehouse & Gatehouse, 1998). Although the serine protease inhibitors participate in reactions controlling protease activities in different physiological processes, their functions in the organism where they are found are not yet fully understood. However, plant protease inhibitors have been described as storage proteins (Xavier-Filho, 1992) and as protective agents against plant infection processes (Ryan, 1991; Shewry & Lucas, 1997; Walker *et al.*, 1997; Purcell *et al.*, 1992; Pernas *et al.*, 2000; Franco *et al.*, 2003). Indeed, plant protease inhibitors have been recognized as being powerful carcinogenesis suppressor agents in animals and *in vivo* transformation systems (Kennedy, 1993, and references cited therein). Moreover, as pointed out by Birk (1993), human populations consuming a large amount of Bowman–Birk inhibitors have been shown to exhibit lower rates of colon, breast, prostate and skin cancers.

The three-dimensional structures of several Bowman–Birk inhibitors in monomeric form (Chen *et al.*, 1992; Suzuki *et al.*, 1993; Werner & Wemmer, 1992; Voss *et al.*, 1996; Song *et al.*, 1999; Li de la Sierra *et al.*, 1999) and in complex with trypsin (Tsunogae *et al.*, 1986; Lin *et al.*, 1993; Li *et al.*, 1994; Koepke *et al.*, 2000) have been reported. All structures of Bowman–Birk inhibitors are similar and have a common fold for the reactive site consisting of two distinct binding domains for serine protease. Each domain is composed of a β-harpin with its loop surface exposed to the solvent in order to bind the active site of the cognate enzyme.

Vigna unguiculata is one of the leguminosae (black-eyed pea, cowpea) of significance for human and animal nutrition in the north-eastern Brazil region and contains large

concentrations of trypsin and/or chymotrypsin inhibitors belonging to the Bowman–Birk family (Xavier-Filho & Ventura, 1988; Gennis & Cantor, 1976). The black-eyed pea trypsin/chymotrypsin inhibitor (BTCI) is a Bowman–Birk-type protease inhibitor isolated from *V. unguiculata* seeds and presents two different and independent reactive sites for trypsin and chymotrypsin (Freitas *et al.*, 1997). The binding of trypsin and chymotrypsin to BTCI was characterized as an endothermic, spontaneous and entropically driven process (Fachetti *et al.*, 1984; Freitas *et al.*, 1999). This inhibitor is a stable globular protein (Silva *et al.*, 2001) with 83 residues and seven disulfide bonds (Morhy & Ventura, 1987). The secondary-structure arrangement of BTCI has been estimated by FT-IR as 32% β-structure, 23% β-turns and 28% unordered structure (Freitas & Ventura, 1996). Spectroscopic data on solvent perturbation and surface accessibility, together with the molecular model obtained by homology simulation, indicate an unusual hydrophobic surface exposure (Xavier-Filho & Ventura, 1988; Freitas *et al.*, 1997). Rao *et al.* (1999) reported the crystallization and preliminary X-ray studies of a serine protease inhibitor from *V. unguiculata*, but the refined structure was not reported or deposited in the PDB. In this paper, we present the crystallization of BTCI in complex with bovine β-trypsin, preliminary characterization of the data collected and its molecular-replacement solution.

2. Materials and methods

BTCI was extracted from *V. unguiculata* seeds (Ventura & Xavier-Filho, 1966) and bovine β-trypsin was purchased from Sigma. The complex was obtained by mixing BTCI and trypsin in equimolar amounts and the complex was purified according to Ventura *et al.* (1975). Crystallization was performed using the hanging-drop vapour-diffusion method (McPherson, 1990) at 293 K. Initial crystallization trials were performed using the commercial screens Crystal Screen and Crystal Screen II from Hampton Research. Further improvement of the initial crystallization conditions was carried out by changing the buffer pH and precipitant concentration. In the optimized crystallization conditions for the BTCI–trypsin complex, crystals were grown in a 4 µl droplet containing 2 µl protein solution at 8 mg ml^{-1} in sodium citrate buffer pH 4.0 and 2 µl reservoir solution consisting of 0.1 *M* Na HEPES buffer at pH 7.5, 5%(*v*/*v*) PEG 400 and 2.0 *M* ammonium sulfate.

Crystallographic data were collected at the protein crystallography beamline D03B-CPR at the Laboratório Nacional de Luz Síncrotron (LNLS), Campinas, Brazil. The wavelength of the beamline was coincidentally set to 1.5418 Å, the Cu $K\alpha$ characteristic radiation. A MAR345 image-plate detector (MAR Research) was used to record the data. Crystals were cryocooled in a stream of nitrogen gas at 110 K to minimize radiation damage. The solution in which the crystals were grown provided partial protection against ice formation. Data processing was carried out using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

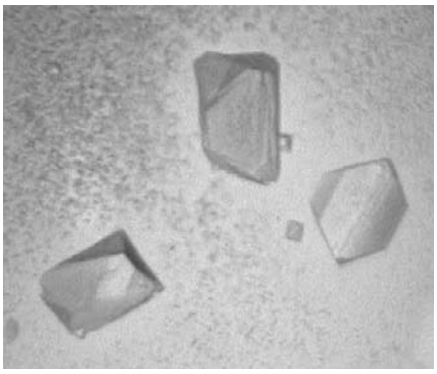

Figure 1
Orthorhombic crystals of the BTCI–trypsin complex, with an average size of 0.4 mm in the longest dimension.

3. Results and discussion

The crystals of BTCI in complex with bovine β-trypsin (Fig. 1) diffracted to approximately 2.3 Å and the diffraction shows the symmetry and systematic absences of the orthorhombic space group $P2_12_12_1$. Data-processing statistics are shown in Table 1. Matthews coefficients (Matthews, 1968) were calculated from the unit-cell parameters, giving a best result of V_M = 2.2 Å^3 Da^{-1} and a solvent content of 42.9%, corresponding to one monomer of the complex per asymmetric unit.

A molecular-replacement solution was found using the crystallographic structure of trypsin in complex with *Phaseolus angularis* Bowman–Birk inhibitor (PDB code 1tab; Tsunogae *et al.*, 1986) as a search model. This procedure was performed with the *CNS* program v.1.1 (Brünger *et al.*, 1998). The rotation-function peak was 7.7σ above the mean, while the translation-function solution was 3.6 times greater than the first incorrect solution. The resulting orientation matrix was applied to the search model, which was then submitted to an initial rigid-body refinement, yielding an R_{cryst} of 0.465 and an R_{free} of 0.471. The model was subjected to a protocol consisting of simulated annealing, positional energy minimization and individual temperature-factor refinement, after which it presented the following R values: R_{cryst} = 0.361 and R_{free} = 0.432. All refinement procedures were also carried out with *CNS*, while the graphics modelling package *O* (Jones *et al.*, 1991) was used for model visualization. Initial electron-density maps were calculated and were of good enough quality to allow the recognition of side chains that differed between the *P. angularis* inhibitor model and the BTCI sequence (Morhy & Ventura, 1987). At present, the model is being constructed and refined.

Table 1
Data-collection statistics.

Values in parentheses are for the last resolution shell (2.44–2.36 Å).

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 59.3, b = 61.8, c = 80.0
Temperature (K)	110
Wavelength (Å)	1.5418
Oscillation (°)	1
Detector distance (mm)	220
No. of frames	290
Resolution limits (Å)	30.00–2.36 (2.44–2.36)
$I/\sigma(I)$ after merging	12.5 (2.7)
Completeness (%)	98.0 (83.0)
R_{sym}	0.137 (0.373)
No. of reflections	103885
No. of unique reflections	12382 (1027)

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