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Crystallization and preliminary X-ray analysis of a novel Kunitz-type kallikrein inhibitor from *Bauhinia bauhinioides*

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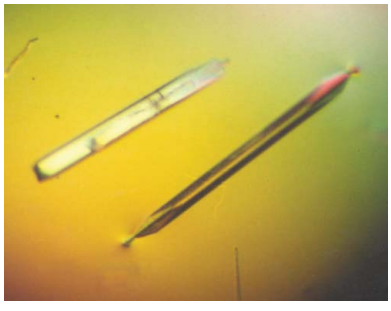

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A Kunitz-type protease inhibitor (BbKI) found in *Bauhinia bauhinioides* seeds has been overexpressed in *Escherichia coli* and crystallized at 293 K using PEG 4000 as the precipitant. X-ray diffraction data have been collected to 1.87 Å resolution using an in-house X-ray generator. The crystals of the recombinant protein (rBbKI) belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 46.70, b = 64.14, c = 59.24 Å. Calculation of the Matthews coefficient suggests the presence of one monomer of rBbKI in the asymmetric unit, with a corresponding solvent content of 51% (V_M = 2.5 Å^3 Da^{-1}). Iodinated crystals were prepared and a derivative data set was also collected at 2.1 Å resolution. Crystals soaked for a few seconds in a cryogenic solution containing 0.5 *M* NaI were found to be reasonably isomorphous to the native crystals. Furthermore, the presence of iodide anions could be confirmed in the NaI-derivatized crystal. Data sets from native and derivative crystals are being evaluated for use in crystal structure determination by means of the SIRAS (single isomorphous replacement with anomalous scattering) method.

1. Introduction

Plant Kunitz-type protease inhibitors present several conserved structural features, such as a molecular weight of approximately 20 kDa, the formation of a 1:1 complex with the target protease and a composition consisting of about 180 amino-acid residues and usually containing four cysteine residues forming two disulfide bridges in conserved positions (Bode & Huber, 1992). Initially, on the basis of its proximity to the reactive site, one of these disulfide bonds [Cys39–Cys83 in *Erythrina* trypsin inhibitor (ETI) numbering] was thought to play an important role in stabilizing the reactive-site loop. However, further investigation has shown that even in the absence of such a bridge, the protein ETI does not lose its inhibitory efficiency toward trypsin. These studies involved both chemical modification of the protein by disulfide-bond reduction (Lehle *et al.*, 1994) and substitution of the cysteines involved in the disulfide bond by site-directed mutagenesis (Lehle *et al.*, 1996). Although these reports indicate that disulfide bridges in Kunitz-type protease inhibitors are not essential for reactive-site loop stabilization, contradictory results were observed in similar experiments with the soy bean trypsin inhibitor (STI), which has been shown to be fully inactivated upon reduction (Steiner, 1965).

Recently, a Kunitz-type protease inhibitor was identified in *Bauhinia bauhinioides* seeds (BbKI; Oliva *et al.*, 1999). It was shown that BbKI is able to specifically inhibit trypsin and plasma kallikrein, probably owing to the arginine residue located at the P1 position in the reactive loop (Oliva *et al.*, 1999, 2001). With regard to the structural features common to the Kunitz-type inhibitor family, several conserved residues are found in the BbKI sequence; for example, Asp5, Gly8, Pro10, Tyr17-Tyr18, Gly28 and Gly35-Asn36-Glu37 (STI numbering; Richardson, 1991). However, unlike the majority of proteins belonging to this family, BbKI lacks both disulfide bonds, containing only one non-conservative cysteine residue at position 154 (Oliva *et al.*, 2001). Furthermore, recombinant

BbKI (rBbKI) has been heterologously expressed in *Escherichia coli* in its active form, as shown by the similar inhibition constants for bovine trypsin and human plasma kallikrein compared with the native protein (Araújo *et al.*, 2005).

The structural elucidation of this new member of the Kunitz-type family, a naturally occurring protease inhibitor devoid of disulfide bridges, could clarify the role of these bridges in protein stabilization and its relationship to the maintenance of inhibitory activity. Here, we report the crystallization and preliminary X-ray diffraction analysis of rBbKI.

2. Material and methods

2.1. Protein expression and purification

The overexpression of N-terminal His-tagged rBbKI in *E. coli* BL21 (DE3) and its subsequent purification were carried out using previously described methods (Araújo *et al.*, 2005). Briefly, cells containing the target gene cloned into the expression vector pET28a (Novagen) were grown in Luria–Bertani medium to an optical density $OD_{600\,nm}$ of 0.5, followed by induction of the fusion-protein expression with 0.2 m*M* IPTG for 4 h at 310 K. Bacteria were lysed by sonication in 50 m*M* Tris–HCl buffer, 150 m*M* NaCl pH 8.0 after a 30 min incubation with 1 mg ml^{-1} lysozyme. The fusion protein was then purified by Ni–NTA affinity chromatography and eluted with an imidazole gradient (100–250 m*M*) in the same sonication buffer. Fractions containing the fusion protein were combined and dialyzed against PBS buffer (137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4 pH 7.5) in order to remove the imidazole and to switch the buffer for subsequent enzyme digestion with 1 U thrombin (GE Healthcare) per milligram of fusion protein for 4 h at 291 K. As a consequence of the introduced thrombin-cleavage site in the expression-vector construct, the rBbKI liberated after hydrolysis contains three non-native amino acids at the N-terminus (Ser-Gly-His). The His tag was then separated from the rBbKI protein by size-exclusion chromatography (Superdex 75 HR 10/30 column; GE Healthcare) in PBS buffer. The fractions containing rBbKI were pooled and concentrated for crystallization experiments.

2.2. Crystallization

Initial crystallization trials were performed by the hanging-drop vapour-diffusion method using sparse-matrix screens (Jancarik & Kim, 1991) based on the Crystal Screen 1 and 2 crystallization kits (Hampton Research) at 293 K. Drops containing 3 µl protein solution (6.5 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl buffer) mixed with the same volume of reservoir solution were equilibrated against 500 µl of the latter. Several conditions based on polyethylene glycol as precipitant in the pH range 4.6–6.5 (solution Nos. 9, 10, 15, 20, 37, 49 and 50 of Crystal Screen 1) produced two morphologically different crystals in approximately one week: clusters of thin plates and bar-shaped crystals. Those grown under condition No. 37 [8%(*w*/*v*) PEG 4000 and 0.1 *M* sodium acetate buffer pH 4.6; Fig. 1] were suitable for X-ray diffraction experiments.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Data set	Native	Iodide derivative
Wavelength (Å)	1.5418 (Cu $K\alpha$)	
Space group	$P2_12_12_1$	
Unit-cell parameters (Å, °)	a = 46.7, b = 64.2, c = 59.2	a = 47.1, b = 63.1, c = 59.8
Average mosaicity (°)	0.5	0.7
Resolution range (Å)	26.44–1.87 (1.97–1.87)	26.17–2.10 (2.21–2.10)
Total rotation (°)	213	250
Total reflections	124902	104472
Unique reflections	14628	10317
Redundancy	8.5 (8.4)	5.5 (5.5)
Completeness (%)	95.8 (91.7)	95.2 (92.9)
Average $I/\sigma(I)$	32.8 (10.7)	22.6 (5.7)
R_{merge}† (%)	4.4 (20.7)	11.6 (45.5)
R_{anom}‡ (%)	—	9.6 (42.3)
Scaling R factor§ (%)	—	33.3 [25.00–2.50 Å]

† R_{merge} for the native data is as follows: $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith intensity measurement of reflection hkl, including symmetry-related reflections, and $\langle I(hkl)\rangle$ is its average. R_{merge} for the iodide-derivative data is defined in the same way as R_{merge} for the native data, except that the summation is executed for separated Bijvoet pairs individually. ‡ $R_{anom} = \sum_{hkl} |\langle I^+(hkl)\rangle - \langle I^-(hkl)\rangle| / |\langle I^+(hkl)\rangle - \langle I^-(hkl)\rangle|$, where $\langle I^+(hkl)\rangle$ and $\langle I^+(hkl)\rangle$ are the averages of $I^+(hkl)$ and $I^-(hkl)$, respectively. § The scaling R factor (R_{iso}) = $\sum_{hkl} ||F_{deriv}(hkl)| - |F_{native}(hkl)|| / \sum_{hkl} |F_{native}(hkl)|$.

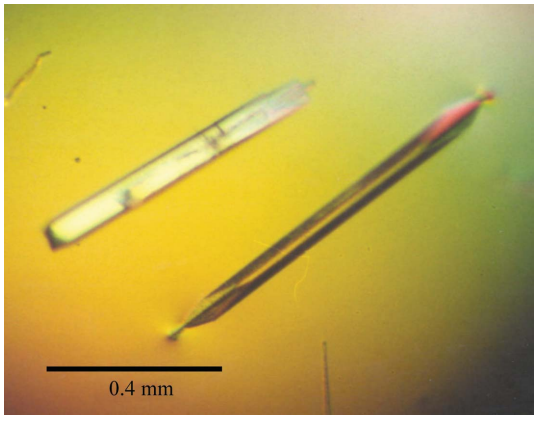

Figure 1
Typical crystals of recombinant BbKI. Crystals were obtained by the hanging-drop vapour-diffusion technique using PEG 4000 as precipitant.

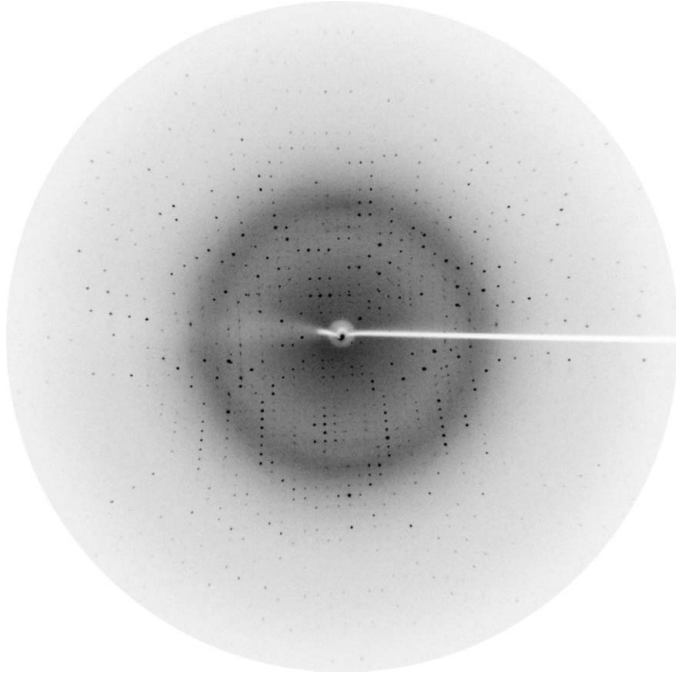

Figure 2
Typical diffraction pattern of the native crystal for 1° oscillation exposure. The edge of the detector corresponds to a resolution of 1.87 Å.

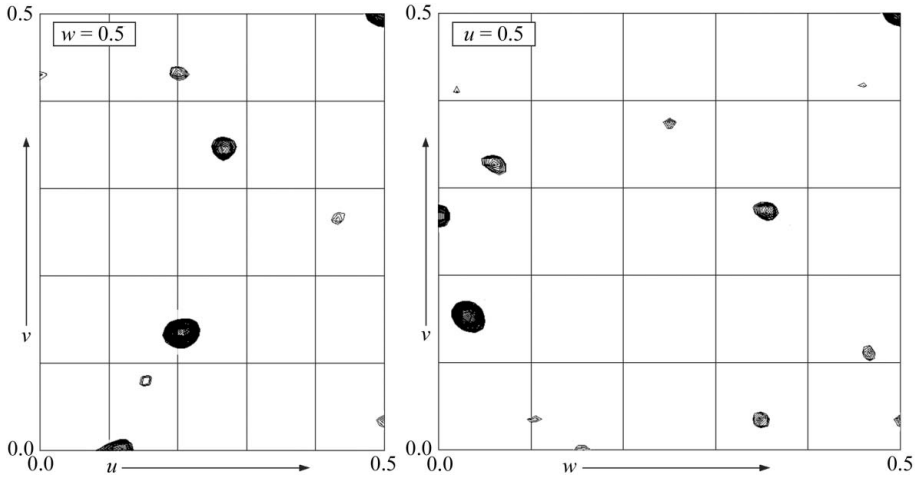

Figure 3
Harker sections (w = 0.5 and u = 0.5) of the anomalous Patterson map of the NaI-derivative BbKI crystal. Levels are contoured in 0.3σ steps starting at 2.4σ.

2.3. Data collection and processing

Single crystals were harvested using nylon loops (Hampton Research) and transferred from the crystallization drop to 10 µl of a cryogenic solution containing the mother liquor and 15%(*v*/*v*) ethylene glycol for a few seconds. The crystals were then flash-cooled to 100 K in a cold nitrogen stream and used for data collection. Native protein data were collected with a rotation range of 1° per image for 3 min on a MAR345 image-plate detector using Cu $K\alpha$ radiation generated from a Rigaku UltraX 18 rotating-anode generator and focused by Osmic mirrors. The crystal-to-detector distance was set to 150 mm and a total of 213 images were collected to a resolution of 1.87 Å (Fig. 2). NaI-derivatized crystals were prepared according to the quick-cryosoaking procedure (Dauter *et al.*, 2000; Nagem *et al.*, 2001). The native crystals were soaked for 30, 60 or 120 s in drops containing 0.25, 0.5 or 1.0 *M* NaI in the same cryogenic solution used for native crystal data collection. The derivative data set used in this work was collected from a single crystal that diffracted X-rays to beyond 2.10 Å resolution even after soaking in the drop containing the highest concentration of NaI. This data set was also collected on a MAR 345 image-plate area detector mounted on a Rigaku UltraX 18 source with Cu $K\alpha$ radiation. The rotation per frame was defined as 1° over a total range of 250 images. The crystal-to-detector distance was set to 180 mm. Both native and derivative data sets were processed using the program *MOSFLM* (Leslie, 1992) and the resulting intensities were scaled and merged using the program *SCALA* (Evans, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The maximum size of the crystals grown under condition No. 37 of Crystal Screen 1 and subsequently used for data collection was approximately 0.9 × 0.1 × 0.05 mm. Assuming the presence of one molecule per asymmetric unit, the calculated Matthews coefficient V_M is 2.5 Å^3 Da^{-1}, which corresponds to a solvent content of 51%. Data-collection statistics are given in Table 1.

Attempts to solve the crystal structure of rBbKI by molecular replacement using the *Erythrina caffra* protease inhibitor (EcTI; Onesti *et al.*, 1991) as a search model (30.2% sequence identity with BbKI) were made. However, none of these attempts provided a clear solution. After several tests employing different soaking times and NaI concentrations, derivative crystals that produced high-quality diffraction patterns to 2.1 Å resolution were obtained using the optimal soaking condition (60 s in a cryogenic solution containing 0.5 *M* NaI). The final data set was 92.5% complete with an R_{anom} of 9.6%. An anomalous Patterson map was calculated using data in the entire resolution range. The resulting u = 0.5 and w = 0.5 Harker sections are shown in Fig. 3. Peaks corresponding to the inter-iodide-ion vectors are clearly observed in these sections. Furthermore, the scaling of the derivative with the native data showed that the NaI derivative was isomorphous to the native crystal, consistent with the small differences in the unit-cell parameters (a = 46.7 + 0.5 Å, b = 64.2 − 0.9 Å, c = 59.2 + 0.6 Å) and the relatively low scaling R factor (Table 1).

In order to proceed with the structure solution, the program *SHELXD* (Sheldrick, 1998) was used to locate the iodide sites in the asymmetric unit of the crystal using the anomalous differences from the derivative data set. Trial runs of *SHELXD* were performed in order to estimate the number of iodide ions which had been incorporated into the crystal during the quick-cryosoaking procedure. Given that crystals derivatized using this technique generally present several sites with decreasing occupancies (Dauter *et al.*, 2000; Nagem *et al.*, 2001), such an estimation was performed based on the refined occupancies of the sites found. In this way, the eight major iodide sites located with occupancies above 20% were used for SIRAS phasing using the program *SHARP* (de La Fortelle & Bricogne, 1997). Experimental phases obtained by *SHARP* were improved by density-modification protocols using the programs *SOLOMON* (Abrahams & Leslie, 1996) and *DM* (Cowtan, 1994). Model building and refinement of the complete rBbKI is currently in progress.

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