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Purification, crystallization and preliminary crystallographic study of a Kunitz-type trypsin inhibitor from *Delonix regia* seeds

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The Kunitz-type trypsin inhibitor from seeds of Flamboyant (*Delonix regia*) has been purified to homogeneity and plate-like crystals suitable for X-ray analysis have been grown by the hanging-drop method using PEG 6000 as a precipitant. The crystals belong to space group $P2_12_12_1$ with unit-cell parameters a = 32.15, b = 69.39, c = 72.54 Å. X-ray diffraction data have been collected to 2.95 Å resolution. The structure has been solved by molecular replacement using the known structures of trypsin inhibitors from *Erythrina caffra* seeds (PDB code 1tie) and from soya beans (*Glycine max*; PDB code 1ba7) as search models.

1. Introduction

Plant serine-proteinase inhibitors have been classified in different families according to the class of the enzyme inhibited, the sequence homology and the number of cysteine residues (for reviews, see Ryan & Walker-Simons, 1981; Rackis *et al.*, 1986; Richardson, 1991; Anderson & Wolf, 1995). Among the most important are the Kunitz and the Bowman–Birk proteinase-inhibitor families, which have been studied in great detail (Ryan & Walker-Simons, 1981; Odani *et al.*, 1986; Richardson, 1991; Tanaka *et al.*, 1997). The Bowman–Birk proteinase inhibitors have molecular weights of 8–10 kDa and a high cystine content (usually seven disulfides). The Kunitz-type proteinase inhibitors have molecular weights of ∼20 kDa and normally only two disulfide bridges. The Kunitz soybean trypsin inhibitor (Koide & Ikenaka, 1973), the inhibitors from *Erythrinia caffra* (Joubert & Dowdle, 1987), winged bean (*Psophocarpus tetraganolobus*; Yamamoto *et al.*, 1983), the seeds of *Albizzia jubibrissin* (Odani *et al.*, 1979), *Erythrina latissima* (Joubert *et al.*, 1981) and *Acacia elata* (Kortt & Jermyn, 1981) are members of this family.

The presence of proteinase inhibitors in living tissues seems to be essential for natural regulatory processes; for example, plants protecting themselves against insect predation (Garcia-Carrenõ, 1996). The stability of proteinase inhibitors towards enzymes as well as the large quantities present in seeds indicate the importance of protease inhibitors as storage proteins (Ryan, 1973). The enzyme inhibitors encountered in seeds can cause nutritional disorders and toxic effects when ingested by animals and humans as components of plant foods. Furthermore, there is an interest in the possible pharmacological use of plant inhibitors in the treatment of a wide range of metabolic disorders associated with enhanced proteolytic activity (pancreatitis, emphysema, allergy, inflammation and certain cancers; Richardson, 1991).

The Flamboyant (*Delonix regia*) tree is distributed in the northern and western parts of Madagascar and is widely cultivated in many tropical countries. Trees of the order *Delonix* (Leguminosae/Caesalpinioideae) are used as ornamental trees and the seeds of some species are crushed and eaten raw as a snack in south Madagascar (Puy *et al.*, 1995). We have used the seeds of Flamboyant as a source of the trypsin inhibitor DrTi, which was purified and used for crystallographic studies.

2. Experimental

2.1. Protein purification

70 g of *D. egia* seeds were milled to a fine powder, homogenized with 150 m*M* NaCl [10%(*w*/*v*)] solution and agitated for 1 h at room temperature. The method for isolation of DrTi was similar to that described by Tanaka *et al.* (1997). Proteins from the crude extract were precipitated at 277 K by slow addition of acetone to a final concentration of 80%(*v*/*v*) and the precipitate was collected by centrifugation (350*g*, 20 min, 277 K). The precipitate was dissolved in 50 m*M* Tris–HCl buffer pH 8.0 (buffer *A*), loaded on a DEAE Sephadex A-50 column (1.5 × 30 cm) equilibrated with the same buffer and eluted with a linear gradient of NaCl (0–0.5 *M*) in buffer *A*. The inhibitor-containing fractions were pooled, dialysed against two 2 l changes of 5 m*M* ammonium bicarbonate and lyophilized. This lyophilized powder was dissolved in 0.15 *M* NaCl in buffer *A* and applied to a Sephadex G-75 column (1.5 × 100 cm) equilibrated with the same solution. Fractions which contained inhibitor activity

were pooled and, finally, DrTi was purified on a DEAE 5 PW column (4.5 × 75 mm, Waters), equilibrated with 20 m*M* Tris–HCl buffer in a linear gradient (0.05–0.5 *M*, 60 min) of NaCl at a flow rate of 1.0 ml min^{-1}. The purified inhibitor was dialysed against 5 m*M* ammonium bicarbonate and lyophilized. Protein elution was monitored at 280 nm. The biological activity of the inhibitor was detected using bovine trypsin and *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as described by Gaertner & Puigserver (1992). The purity of DrTi was estimated to be 95% by SDS–PAGE in gradient gel (10–20%). The identity of the protein has been confirmed by N-terminal amino-acid sequencing.

2.2. Crystallization

DrTi was crystallized by vapour diffusion in hanging drops at 291 K (McPherson, 1982). The inhibitor was dissolved in 25 m*M* potassium phosphate buffer to a final concentration of 22 mg ml^{-1}. 10 µl of this solution was mixed with an equal amount of 7.5% PEG 6000 in 12 m*M* potassium phosphate buffer in the pH range 5.4–6.3. After centrifugation (4000*g*, 5 min), 18 µl drops of the resulting solution were equilibrated against 1 ml of reservoir solution containing 15% PEG 6000 in 100 m*M* potassium phosphate buffer pH 6.0. Crystals of DrTi appeared after about one month and had average dimensions of 0.1 × 0.1 × 0.05 mm.

2.3. Data collection and processing

X-ray data from a single native crystal were collected at room temperature at the protein crystallography beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) of the National Synchrotron Light Laboratory (LNLS, Campinas, Brazil) using a 345 mm MAR Research imaging-plate detector and monochromatic X-ray radiation of wavelength 1.38 Å. 96 oscillation images were collected, with an angular step of 1° and an exposure of 30000 dose units per image. The DrTi crystal, which initially diffracted beyond 2.5 Å resolution, showed significant radiation decay during data collection. Data were processed with *DENZO* and *SCALEPACK* (Otwinowski, 1993) and final processing was limited to 2.95 Å resolution.

3. Results and discussion

DrTi crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell dimensions a = 32.15, b = 69.39, c = 72.54 Å. The final data set is 89.3% complete between 13 and 2.95 Å resolution, with an overall $R_{\rm merge}$ of 8.2% ($R_{\rm merge}$ in the last resolution shell between 2.95 and 3.0 Å is 29.8%). Completeness within the last resolution shell is 96.0%. A total of 8199 reflections have been collected, out of which 3326 reflections were unique.

Assuming a molecular weight of 21.1 kDa and a single DrTi molecule per asymmetric unit cell, the Matthews V_m value is 1.92 Å^3 Da^{-1} (Matthews, 1968). Based on this value, estimates of the solvent content of the crystal and the crystal density are 36% and 1.2 g cm^{-3}, respectively (Drenth, 1994). This information indicated the presence of a single DrTi molecule in the asymmetric unit, which was confirmed by the molecular-replacement structure solution.

The DrTi structure was solved by the molecular-replacement method with the program *AMoRe* (Navaza, 1994), using existing crystallographic models of Kunitz-type trypsin inhibitors from soya beans (STI; De Meester *et al.*, 1988) and from *Erythrina caffra* seeds (ETI; Onesti *et al.*, 1991).

The rotation function calculated for the STI search model (PDB code 1ba7), using diffraction data in the resolution range 10–2.95 Å and a Patterson radius of 20 Å, resulted in a first solution with a correlation coefficient (CC) of 21.5%, whereas the second and third solutions had CCs of 14.2 and 14.1%, respectively. A translation search performed with the same program, using the Crowther & Blow (1967) translation function, resulted in a clear solution with a CC of 48.7% and an *R* factor of 50.6%. The second translation-function solution had a CC of 39.4% and an *R* factor of 53.4%, and the third had a CC of 39.2% and an *R* factor of 54.0%. The model was finally subjected to rigid-body refinement in the resolution range 10–2.95 Å, which slightly improved both the *R* factor and the correlation coefficient. The resulting CC was 48.9% and the *R* factor was 50.3%.

An equally clear solution was found using the 2.5 Å resolution structure of the trypsin inhibitor from *Erythrina caffra* seeds (PDB code 1tie). The same resolution range and Patterson radius were used in the calculations. The rotation peak corresponding to the correct solution was the highest (correlation coefficient of 23.4%). The second and the third solutions had correlation coefficients of 12 and 11.7%, respectively. The first solution of the translation search had a CC of 43.0% and an *R* factor of 52.7%. The second translation function solution had a CC of 35.5% and an *R* factor of 55.2%, and the third had a CC of 35.3% and an *R* factor of 55.3%. The model was finally subjected to rigid-body refinement in the resolution range 10–2.95 Å, which slightly improved both the *R* factor and the correlation coefficient. The resulting CC was 44.1% and the *R* factor was 52.3%. Both search models resulted in essentially the same structure solution.

Preliminary refinement of both the ETI and STI models was undertaken using the program *REFMAC* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). After 15 cycles of positional and overall *B*-factor refinement of the final molecular-replacement solutions, electron-density maps were calculated and averaged, with the objective of minimizing the phase errors associated with model bias and the incomplete amino-acid sequence. The model has been rebuilt to match the averaged electron density. The primary sequence has been mutated according to the available DrTi partial primary sequence (Tanaka *et al.*, 1997). Further steps of positional and isotropic *B*-factor refinement have been undertaken. The current *R* and $R_{\rm free}$ are 29.3 and 39.4%, respectively. The complete primary sequence of DrTi is currently being determined by amino-acid sequencing. Further work on model rebuilding and refinement is under way.

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