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Purification, crystallization and X-ray characterization of a Kunitz-type trypsin inhibitor protein from the seeds of chickpea (*Cicer arietinum*)

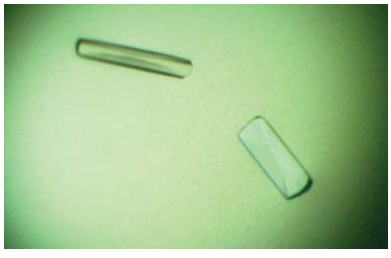
A Kunitz-type trypsin inhibitor protein (CPTI) purified from chickpea seeds was estimated to have a molecular mass of 18 kDa on SDS–PAGE. The IC_{50} value of CPTI was determined to be 2.5 µg against trypsin. The inhibitory activity of CPTI is 114 TIU (trypsin inhibitory units) per milligram of protein, which is high compared with those of other known Kunitz-type trypsin inhibitors from legumes. CPTI crystallized in three different orthorhombic crystal forms: $P2_12_12$ form *A*, $P2_12_12$ form *B* and $P2_12_12_1$. The crystals of $P2_12_12$ form *A*, with unit-cell parameters a = 37.2, b = 41.2, c = 104.6 Å, diffracted to 2.0 Å resolution at the home source and to 1.4 Å on beamline BM14 at the ESRF. Data were also collected from crystals grown in the presence of iodine. The Matthews coefficient for these crystals was calculated to be 2.37 $Å^3$ Da^{-1}, corresponding to a solvent content of 42%. The other two crystal forms ($P2_12_12$ form *B* and $P2_12_12_1$) diffracted comparatively poorly.

1. Introduction

Protease inhibitors (PIs) are synthesized by plants as a defence against pathogens and are the most studied class of plant defence proteins. PIs in plants are considered to be antinutritional factors. They have been implicated in various physiological functions such as regulation of proteolysis, as storage proteins and as defence molecules against plant pests and pathogens (Koiwa *et al.*, 1997; Ryan, 1990). The interaction of most PIs with their target proteases results in the formation of a stable protease–inhibitor complex. PIs are divided into families depending on the proteolytic enzymes inhibited. They share extensive sequential and structural homology as well as conserved disulfide bridges and reactive sites (Laskowski & Kato, 1980). The four groups of PIs are the serine, cysteine, aspartic and metalloprotease inhibitors (Laskowski & Qasim, 2000).

Protease inhibitors, especially serine protease inhibitors, show enormous diversity in regulating the proteolytic activity of their target proteases (Leung *et al.*, 2000). They have been isolated and characterized extensively from various leguminous plants (Giri *et al.*, 2003). Two types of serine PIs have been characterized by biochemical methods: Kunitz-type (Odani & Ikenaka, 1973) and Bowman–Birk-type (Birk, 1985). The newly identified trypsin inhibitor from chickpea seeds (CPTI) reported here belongs to the Kunitz soybean trypsin inhibitor (KSTI) family, members of which have a molecular mass of about 18–20 kDa and contain two disulfide bridges and a single reactive site for trypsin.

CPTI also showed efficient inhibitory action for chymotrypsin. A Kunitz-type inhibitor of molecular mass 20 kDa has previously been reported from chickpea (*Cicer arietinum*; Srinivasan *et al.*, 2005) which showed inhibitory activity exclusively for trypsin and HGP (*Helicoverpa armigera* gut proteases). A recently characterized trypsin inhibitor from *Cocculus hirsutus*, belonging to the Menispermeaceae family, inhibits bovine trypsin and HGP and had a molecular mass of 18 kDa (Bhattacharjee *et al.*, 2009). Ye & Ng (2002) have also reported a 18 kDa chickpea protein (CLAP) that



resembles cyclophilin-like proteins and possesses antifungal and anti-HIV-1 reverse transcriptase activities. A recently reported leguminous trypsin–chymotrypsin inhibitor, limenin, with a molecular mass of 18 kDa from *Phaseolus limensis* showed antibacterial, antifungal and antiproliferative activities (Wang & Rao, 2010). The CPTI inhibitor reported here has a molecular mass (18 kDa) similar to that of limenin from *P. limensis* and inhibits trypsin and chymotrypsin. The biological roles of all of the Kunitz-type inhibitors mentioned above have been characterized; however, no structure–function studies have been reported for the 18 kDa Kunitz-type inhibitors from legumes. This communication is the first report of the crystallization of a Kunitz-type trypsin inhibitor isolated from chickpea.

2. Materials and methods

2.1. Purification of CPTI

50 g dry seeds of *C. arietinum* cv. PUSA-256 were soaked in 250 ml 10 m*M* Tris–HCl pH 7.2 containing 150 m*M* NaCl. The soaked seeds were homogenized in a blender and the suspension was stirred overnight, filtered through a muslin cloth to remove coarse particles and centrifuged at 11 000 rev min^{-1} for 20 min at 277 K. The supernatant was filtered through Whatman filter paper 1 to remove the lipidic suspension. The filtrate was subjected to acid precipitation at pH 4.5 and subsequently centrifuged at 11 000 rev min^{-1} for 20 min at 277 K. The supernatant was again filtered through Whatman filter paper 1 to remove the lipidic suspension. The pH of the filtrate was adjusted back to 7.2 and the filtrate was centrifuged. The supernatant was subjected to 80% saturated ammonium sulfate precipitation. The precipitate was dissolved in 20 m*M* Tris–HCl buffer pH 8.0, dialysed against the same buffer and centrifuged. The clear supernatant was loaded onto a DEAE-cellulose column pre-equilibrated with 20 m*M* Tris–HCl buffer pH 8.0. The protein was obtained in the unadsorbed fractions. These fractions were resolved on 12% SDS–PAGE and were also checked for activity. Fractions containing the 18 kDa CPTI were pooled together, dialysed against 20 m*M* acetate buffer pH 5.0 and loaded onto an SP-Sephadex column pre-equilibrated with 20 m*M* acetate buffer pH 5.0. Elution of the bound protein was achieved using a stepwise 0.1–0.5 *M* NaCl gradient; the CPTI eluted between 0.2 and 0.3 *M* NaCl. All eluates were resolved on 12% SDS–PAGE. The purified and active fractions of CPTI were stored at 253 K for further use.

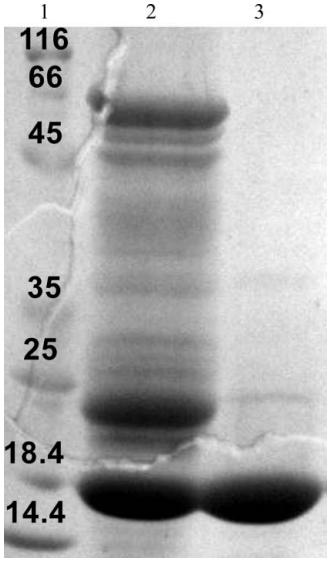


Figure 1
SDS–PAGE (12%) of purified CPTI shows a single band of molecular mass 18 kDa. Lane 1, molecular-weight markers (Fermentas; labelled in kDa); lane 2, DEAE unadsorbed fraction; lane 3, SP Sephadex fraction eluted with 200 m*M* NaCl.

2.2. Protease inhibitory activity of CPTI

Trypsin-like activity was estimated using the enzyme-specific chromogenic substrate BAPNA as reported elsewhere (Giri *et al.*, 1998). Trypsin activity was first calibrated by end-point titration of varying amounts of protease (5, 10 and 25 µg bovine trypsin). For the BAPNA assay, 150 µl diluted enzyme solution was added to 1 ml 1 m*M* substrate solution (BAPNA in 0.1 *M* Tris buffer pH 7.8) and incubated at 310 K for 10 min. The reaction was terminated by the addition of 200 µl 30% acetic acid and the absorbance of BAPNA was checked at 410 nm.

Chymotrypsin inhibition activity was measured using the azocaseinolytic assay (Brock *et al.*, 1982). To carry out the azocaseinolytic assay, 60 µl diluted enzyme was added to 200 µl 1% azocasein in 0.2 *M* glycine–NaOH pH 10.0 and incubated at 310 K for 30 min. The reaction was terminated by the addition of 300 µl 5% trichloroacetic acid. After centrifugation at 14 230*g* for 10 min at 277 K, an equal volume of 1 *M* NaOH was added to the supernatant and the absorbance was measured at 450 nm. One protease unit was defined as the amount of enzyme that increased the absorbance by 1.0 OD under the given assay conditions. For the inhibitor assay, suitable amounts of inhibitor and enzyme were pre-incubated at room temperature for 20 min and the residual enzyme activity was assayed as above. One PI unit is defined as the amount of inhibitor required to inhibit one protease activity unit.

2.3. Crystallization

Initial crystallization trials were conducted with commercially available crystallization kits supplied by Hampton Research (USA) and Molecular Dimensions Ltd (UK) using the hanging-drop vapour-diffusion method (McPherson, 1982). The purified and active CPTI crystallized from a 25 mg ml^{-1} solution. Crystals appeared and grew to full size in 3 d; these crystals were used for data collection. Attempts were made to prepare a heavy-atom derivative of CPTI using cocrystallization and quick-soaking approaches. For cocrystallization, freshly prepared heavy-metal salts in the concentration range 1–5 m*M* were mixed with protein solution prior to setting up crystallization experiments. Alternatively, pre-formed crystals of CPTI were soaked in mother liquor containing heavy metals at concentrations that the crystals were able to withstand (10–1000 m*M*). The soaking time was varied from 10 s to several hours. Salts of various heavy metals were used, namely $HgCl_2$, mercury(II) acetate, $Pb(NO_3)_2$, KI, NaI, *p*-hydroxymercury benzoate (PCMB), dichloro(ethylenediamine)platinum(II) and sodium molybdate.

2.4. X-ray data collection and analysis

Initial characterization of the CPTI crystals was carried out at room temperature (295 K) using Cu $K\alpha$ radiation from a Rigaku rotating-anode X-ray generator and an R-AXIS IV^{++} image-plate detector. The crystal-to-detector distance was adjusted to 150 mm and each oscillation frame was recorded with 0.5° oscillation. Initial data collected at 2 Å resolution were processed using the *HKL* suite (Otwinowski & Minor, 1997). High-resolution data (1.4 Å) were collected from a CPTI crystal belonging to the orthorhombic $P2_12_12$ form *A* on beamline BM14, ESRF, Grenoble. Diffraction data were recorded on a MAR CCD detector with a diameter of 130 mm. The

crystal-to-detector distance was 100 mm, with 1° oscillation per frame and an exposure time of 10 s. Data processing and scaling were accomplished with *MOSFLM* (Leslie, 1992) and *SCALA* (Evans, 1993) from the *CCP*4 suite (Winn *et al.*, 2011). Data for MAD phasing were collected on the BM14 beamline at the ESRF, Grenoble, France for native CPTI and the iodine derivative at wavelengths of 1.77 and 1.54 Å for sulfur and iodine, respectively; the high-resolution data were collected at a wavelength of 0.95 Å.

3. Results and discussion

The total yield of pure CPTI after the final purification step was 50 mg per 100 g of dried chickpea seeds. Purified and homogeneous CPTI was eluted from the SP Sephadex column with a 200–300 m*M* NaCl gradient. The purified fractions of CPTI were resolved on 12% SDS–PAGE, on which CPTI showed a single band corresponding to a molecular mass of 18 kDa (Fig. 1). Efforts to determine the N-terminal sequence were not successful and hence the degree of similarity between this protein and other known trypsin inhibitors from legumes could not be ascertained.

The inhibitory action of CPTI was determined towards trypsin as well as chymotrypsin. The inhibitory activity was stronger towards trypsin compared with chymotrypsin. The CPTI showed an IC_{50} value of 2.5 µg towards trypsin, while the IC_{50} was 3.0 µg towards chymotrypsin (Figs. 2*a* and 2*b*). The minimum inhibitory concentrations were estimated as 0.8 and 0.75 µg ml^{-1} for trypsin and chymotrypsin, respectively. The corresponding estimated inhibitory activities of

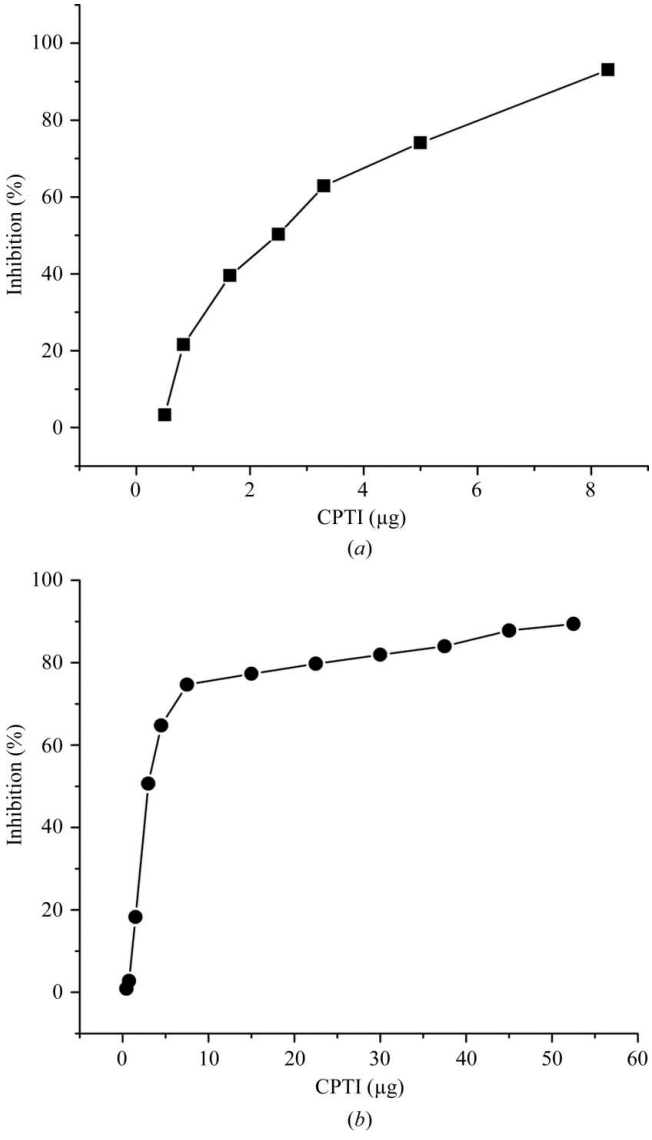


Figure 2
Assay of the inhibitory activity of chickpea trypsin inhibitor (CPTI) towards trypsin (*a*) and chymotrypsin (*b*).

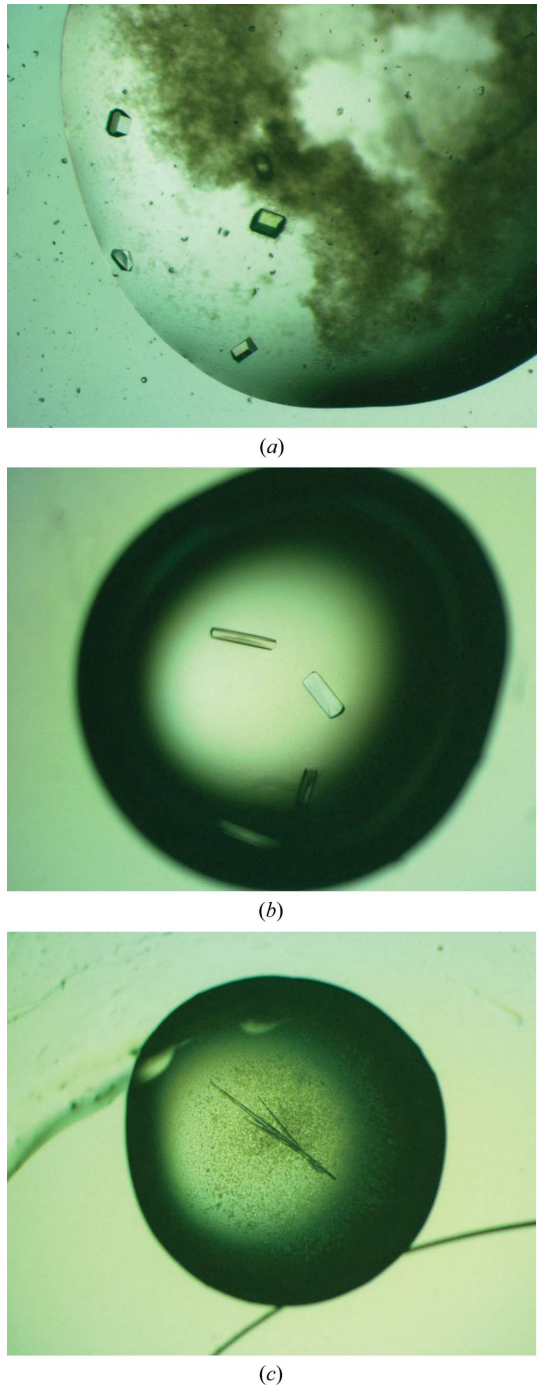


Figure 3
Crystals of the three forms of CPTI. (*a*) Orthorhombic $P2_12_12$ form *A*, with unit-cell parameters $a = 37.2$, $b = 41.2$, $c = 104.6$ Å, (*b*) orthorhombic $P2_12_12_1$ form, with unit-cell parameters $a = 41.1$, $b = 50.1$, $c = 75.1$ Å, and (*c*) orthorhombic $P2_12_12$ form *B*, with unit-cell parameters $a = 44.4$, $b = 75.7$, $c = 133.7$ Å.

Table 1
Data-collection and processing statistics for native and derivatized crystals of CPTI in two orthorhombic forms ($P2_12_12$ form *A* and $P2_12_12_1$).

Values in parentheses are for the last shell.

CPTI crystal	Native ($P2_12_12$ form *A*)	Native ($P2_12_12_1$)	NaI derivative (500 m*M*) ($P2_12_12$ form *A*)
X-ray source	BM14, ESRF	Rotating anode	BM14, ESRF
Space group	$P2_12_12$	$P2_12_12_1$	$P2_12_12$
X-ray wavelength (Å)	0.95	1.5418	0.95
Temperature (K)	93	295	93
Crystal-to-detector distance (mm)	187.6	150	109.4
Mosaicity (°)	0.55	0.40	0.39
No. of frames	720	200	720
Unit-cell parameters			
a (Å)	36.56	41.10	36.48
b (Å)	102.49	50.10	102.50
c (Å)	40.55	75.0	40.43
Unit-cell volume (Å^3)	151942	155156	151175
Resolution range (Å)	34.43–1.60 (1.69–1.60)	50–3.00 (3.05–3.00)	20.50–1.30 (1.37–1.30)
Total reflections	518340	25178	535016
Unique reflections	20675	3400	38120
Multiplicity	25.1	7.25	14
Completeness (%)	99.1 (93.9)	99.9 (98.0)	99.8 (99.4)
$R_{\rm merge}$	0.062 (0.387)	0.119 (0.298)	0.048 (0.403)
Matthews coefficient (Å^3 Da^{-1})	2.11	2.14	2.10
Solvent content (%)	42	43	41

CPTI were 114 TIU (trypsin inhibitory units) per milligram of protein and 77 CIU (chymotrypsin inhibitory units) per milligram of protein, respectively.

Crystallization experiments using purified CPTI yielded three different orthorhombic crystal forms in three different conditions from the JCSG+ screen. Preliminary X-ray characterizations of all three crystal forms were carried out at the home source. Crystals of the orthorhombic $P2_12_12$ form *A*, with unit-cell parameters a = 37.2, b = 41.2, c = 104.6 Å, were obtained from condition No. 1.18 [0.1 *M* citrate–phosphate buffer pH 4.2, 40% ethanol, 5%(*w*/*v*) PEG 1K] of the JCSG+ screen (Fig. 3*a*). The calculated Matthews coefficient assuming the presence of one molecule per asymmetric unit was 2.11 Å^3 Da^{-1} and corresponds to a solvent content of 42% (Table 1). The crystals grew to maximum size in 3–4 d, diffracted to 2.0 Å resolution at the home source and could easily be reproduced. Further studies were carried out with this crystal form only.

Crystals belonging to the orthorhombic $P2_12_12_1$ form, with unit-cell parameters a = 41.1, b = 50.1, c = 75.1 Å (Fig. 3*b*), were obtained from condition No. 2.12 [0.1 *M* imidazole pH 8.0, 10%(*w*/*v*) PEG 8K] of the JCSG+ screen. They only diffracted to 3.0 Å resolution at the home source at room temperature. The crystals of this form could not withstand heavy-atom soaking and their diffraction quality worsened when they were frozen at low temperature. Data-collection and processing parameters for this form are shown in Table 1. Thin needle-shaped crystals of the orthorhombic $P2_12_12$ form *B* were obtained from condition No. 1.9 (0.2 *M* ammonium chloride, 20% PEG 3350) of the JCSG+ screen with unit-cell parameters a = 44.4, b = 75.7, c = 133.7 Å and only diffracted to 3.5 Å resolution (Fig. 3*c*). Complete X-ray diffraction data could not be collected owing to radiation damage.

Although models suitable for structure determination by molecular replacement could not be identified owing to a lack of sequence information, the structures of several other Kunitz-type inhibitors were used for phasing. The solutions of molecular-replacement calculations using *Phaser* (McCoy, 2007) gave poor *Z* scores and thus could not proceed further. Efforts were made to obtain suitable heavy-atom derivatives for structure determination using the MIR, MAD or SAD methods. Data-collection statistics for native and derivatized crystals of the orthorhombic $P2_12_12$ form *A* on the BM14 beamline are shown in Table 1. Using the data sets collected at the synchrotron source, only a low-occupancy site could be characterized for the iodine derivative. We are currently focusing on various ways to phase the reflections in order to solve the crystal structure of this protein inhibitor.

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