Acta Crystallographica Section F
**Structural Biology and Crystallization Communications**
ISSN 1744-3091

# Cloning, purification, crystallization and preliminary X-ray crystallographic analysis of a cyclophilin A-like protein from *Piriformospora indica*

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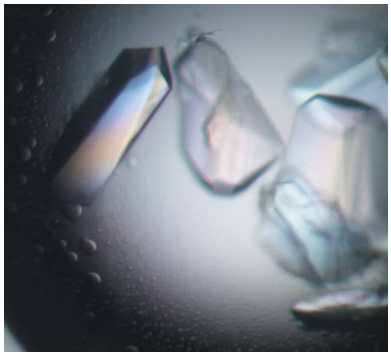
Received 15 March 2012
Accepted 23 April 2012





Cyclophilins are widely distributed both in eukaryotes and prokaryotes and have a primary role as peptidyl-prolyl *cis–trans* isomerases (PPIases). This study focuses on the cloning, expression, purification and crystallization of a salinity-stress-induced cyclophilin A (CypA) homologue from the symbiotic fungus *Piriformospora indica*. Crystallization experiments in the presence of 56 m*M* sodium phosphate monobasic monohydrate, 1.34 *M* potassium phosphate dibasic pH 8.2 yielded crystals that were suitable for X-ray diffraction analysis. The crystals belonged to the orthorhombic space group $C222_1$, with unit-cell parameters $a$ = 121.15, $b$ = 144.12, $c$ = 110.63 Å. The crystals diffracted to a resolution limit of 2.0 Å. Analysis of the diffraction data indicated the presence of three molecules of the protein per asymmetric unit ($V_M$ = 4.48 Å$^3$ Da$^{-1}$, 72.6% solvent content).

## 1. Introduction

Salinity is a severe obstacle to the cultivation of many crop plants in large parts of the world. Overall, salinity stress has become a major threat to plant growth and productivity (Tuteja, 2007). According to a soil map of the world prepared by FAO/UNESCO, approximately 3 230 000 km$^2$ of land worldwide is affected by salinity problems (Brinkman, 1980). *Piriformospora indica*, a plant-root-colonizing basidiomycete fungus, was discovered in the Indian Thar desert and has been shown to provide strong growth-promoting activity during its symbiosis with a broad spectrum of plants (Verma *et al.*, 1998). The fungus interacts with the roots of many plant species and promotes growth, development and seed production and confers resistance to various biotic and abiotic stresses. Salt-stress studies have shown positive effects of *P. indica* on barley (Waller *et al.*, 2005). *P. indica* provides resistance towards fungal diseases and tolerance to salt stress in monocotyledonous plants. We have previously demonstrated the role of *P. indica* and its phosphate-transporter gene (*PiPT*) in the supply of phosphate nutrition to the host plant (Yadav *et al.*, 2010). In addition, we have recently reported that *P. indica* leads to early flowering, higher biomass and altered secondary metabolites in the medicinal plant *Coleus forskohlii* (Das *et al.*, 2012). We have found higher expression levels of a cyclophilin A (CypA) homologue in *P. indica* under salt-stress conditions (unpublished work). This observation is similar to the previously reported role of cyclophilin in conferring salt-stress tolerance in rice (Ruan *et al.*, 2011). Cyclophilins are proteins that are widely distributed and abundantly found in eukaryotic and prokaryotic systems and are present in the cytosol as well as in the nucleus (Wang & Heitman, 2005). Extensive studies of various model organisms have suggested that cyclophilins are involved directly or indirectly in a wide range of cellular processes, including cell division (Schreiber, 1991), transcriptional regulation (Shaw, 2002), protein trafficking (Price *et al.*, 1994), cell signalling (Freeman *et al.*, 1996; Duina *et al.*, 1996), pre-mRNA splicing (Horowitz *et al.*, 2002), molecular chaperoning (Schmid, 1993; Weisman *et al.*, 1996) and stress tolerance (Andreeva *et al.*, 1999; Dominguez-Solis *et al.*, 2008). They are known to possess peptidyl-prolyl *cis–trans* isomerase (PPIase) enzymatic activity, a reaction that

is believed to be involved in the late stages of protein folding (Takahashi *et al.*, 1989; Göthel & Marahiel, 1999). The molecular mechanism of PPIase activity in human T cells has been characterized structurally as well as biochemically (Davis *et al.*, 2010). CypA from human T cells has a high affinity for the immunosuppressive drug cyclosporin A (CsA; Handschumacher *et al.*, 1984) and its PPIase activity can be completely inhibited by CsA. CypA has been shown to interact with calcineurin directly and to modulate $Ca^{2+}$ signalling in human T cells (Liu *et al.*, 1991); $Ca^{2+}$ is a primary signalling molecule in a majority of cellular events and responses. In plants, CypA is involved in signal transduction for various abiotic stresses involving phosphoprotein cascades that are directly regulated *via* $Ca^{2+}$ and other secondary signalling molecules (Xiong *et al.*, 2002). In the present study, we report the cloning, overexpression, purification and crystallization of a CypA homologue from *P. indica* (PiCypA). The study is likely to help in understanding the molecular mechanism(s) involved in the salt-stress response and the role played by PiCypA.

## 2. Experimental methods

### 2.1. Identification of the *cypA* gene of *P. indica*

A cDNA library was constructed from 5 µg poly(A)+ RNA (isolated from *P. indica* grown in 0.6 *M* NaCl) in Uni-ZAP XR vector using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, California, USA). Using an *in vivo* excision system, the cDNA library was converted to phagemids and transferred into *Escherichia coli* SOLR cells. pBluescript SK (pBSK) plasmids containing cDNA inserts were mass-excised from phage stock of the *P. indica* cDNA library using ExAssist helper phage and propagated in *E. coli* SOLR cells. cDNAs of *P. indica* were cloned downstream of the *lac* promoter of the pBSK plasmids, allowing expression of recombinant proteins upon induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). Over one million recombinant *E. coli* cells from the same bacterial culture were plated on Luria–Bertani (LB) agar containing 50 µg ml$^{-1}$ kanamycin, 50 µg ml$^{-1}$ ampicillin, 1 m*M* IPTG and 0.6 *M* NaCl (a concentration that does not permit host bacterial growth). The plates were incubated at 310 K for 12–16 h as described previously (Joshi *et al.*, 2009). 36 bacterial colonies were able to grow on LB plates supplemented with 1 m*M* IPTG and 0.6 *M* NaCl at 310 K. These colonies were plated onto the same medium to confirm their ability to tolerate a high concentration of salt (0.6 *M* NaCl). *E. coli* cells with pBSK vector were used as negative controls. To further confirm the effective contribution of fungal cDNAs to bacterial survival in NaCl and to exclude any association of the observed phenotype with unpredictable chromosomal mutations, the plasmids were purified from the overexpressing colonies of *E. coli* SOLR cells, reintroduced into *E. coli* strain DH5α and re-plated on LB plates containing 1 m*M* IPTG and 0.6 *M* NaCl. Plasmids from these 36 positive colonies (*E. coli* strain DH5α) were sequenced on both strands using Sequenase v.2.0 (US Biochemicals, Cleveland, Ohio, USA). Clones of the expression library were found in frame with the *LacZalfa* gene, which drives expression in the pBSK plasmid. Sequences were compared with the GenBank database using *BLAST N* or *BLAST X* (http://blast.ncbi.nlm.nih.gov/). One of the clones, a cyclophilin A-like protein (accession No. GQ214003), was selected for further studies, as cyclophilins are known to play major roles in $Ca^{2+}$-mediated cell-signalling events in the majority of phyla.

### 2.2. Cloning of PiCypA in expression vector

The entire gene sequence of the cyclophilin-like protein cloned into the pBSK vector was amplified using the following PCR primers: forward, 5′-CTCGAGCATATGTCCCAGCCCAACGTCTACTTTG-3′; reverse, 5′-GAATTCTTAGACAGTGCCAGACGCAGTAATCTTG-3′. Purified PCR product was cloned into the pGEMT-easy vector (Promega) and sequenced using the T7 and SP6 primers, respectively. Subsequent subcloning was performed into the pET-28a vector (Novagen) using *Nde*I and *Eco*RI restriction sites, generating the pET-28a-PiCypA construct.

### 2.3. Expression and purification of PiCypA

The pET28a-PiCypA construct was transformed into *E. coli* BL21 (DE3) Codon Plus cells. Transformed cells were grown in LB medium at 310 K with continuous shaking at 175 rev min$^{-1}$. The cells were induced at an $OD_{600}$ of ∼0.8 using 0.5 m*M* IPTG at 291 K and were harvested after 10 h by centrifugation at 5000*g* for 20 min. The cell pellet was resuspended in lysis and wash buffer (1 *M* NaCl, 25 m*M* imidazole, 25 m*M* phosphate buffer pH 8) and lysed by sonication. The cell lysate was centrifuged at 20 000*g* for 1 h to clear the cell debris. The soluble fraction was transferred onto 5 ml Ni–NTA beads (Qiagen) on a gravity-flow column (GE Healthcare) pre-equilibrated with lysis and wash buffer. Elution of PiCypA was carried out using a five-step gradient of imidazole (25, 50, 100, 200 and 500 m*M*) in the

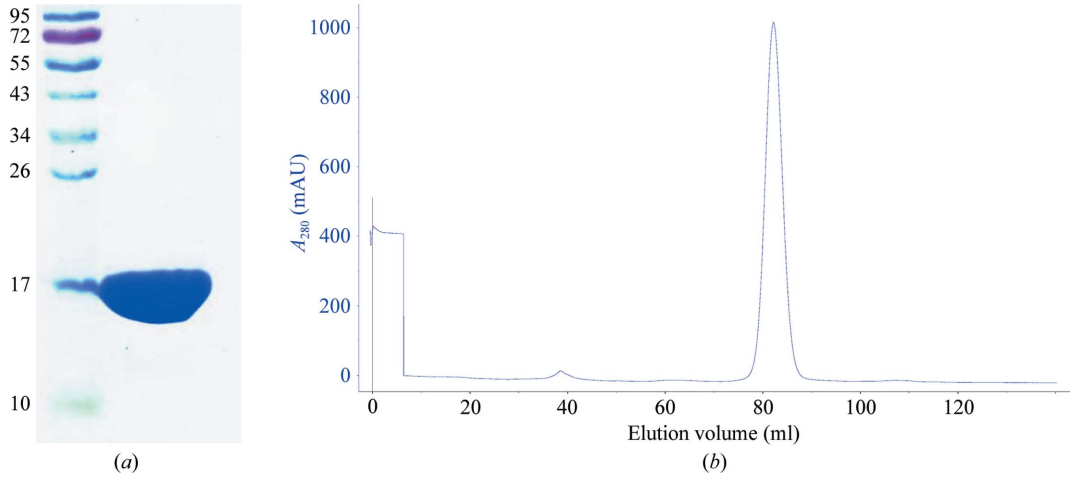



**Figure 1**
(*a*) SDS–PAGE (18%) showing the electrophoretic profile of PiCypA. Lane 1, molecular-mass marker (labelled in kDa); lane 2, PiCypA after gel-filtration chromatography. (*b*) Chromatogram showing the elution profile of PiCypA in gel-filtration chromatography

presence of 1 *M* NaCl and 25 m*M* phosphate buffer pH 6.5. PiCypA eluted in the 200 and 500 m*M* imidazole fractions. Eluates were pooled together and the buffer was exchanged against 50 m*M* NaCl, 20 m*M* Tris–HCl buffer pH 6.5. Further purification of PiCypA was carried out by size-exclusion chromatography on a HiLoad 16/60 Superdex 75 preparative-grade column (GE Healthcare). The purified fraction was concentrated to ∼1 m*M* (18 mg ml$^{-1}$) using a 3 kDa cutoff Centricon (Millipore).

### 2.4. Protein crystallization

Initial crystallization trials were carried out using ∼1 m*M* PiCypA in 50 m*M* NaCl, 20 m*M* phosphate buffer pH 6.5 with sparse-matrix crystallization screens (Hampton Research) at 293 K. Screening was carried out by the sitting-drop vapour-diffusion method with drops consisting of 150 nl protein solution mixed with 150 nl reservoir solution. The crystallization condition was subsequently further optimized manually *via* the hanging-drop vapour-diffusion method with drops consisting of 1 µl protein solution mixed with 1 µl reservoir solution. Diffraction-quality crystals belonging to an orthorhombic space group were grown from a condition consisting of 1 m*M* PiCypA, 56 m*M* sodium phosphate monobasic monohydrate, 1.34 *M* potassium phosphate dibasic pH 8.2

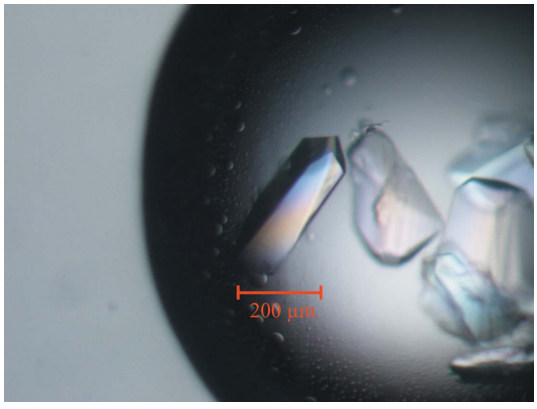



**Figure 2**
Morphology of the PiCypA crystal. The approximate dimensions of the orthorhombic crystals were 200 × 75 × 55 µm.

**Table 1**
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | $C222_1$ |
| Unit-cell parameters (Å, °) | $a$ = 121.15, $b$ = 144.12, $c$ = 110.63, $\alpha = \beta = \gamma$ = 90 |
| Matthews coefficient (Å$^3$ Da$^{-1}$) | 4.48 |
| Solvent content (%) | 72.6 |
| Data-collection temperature (K) | 100 |
| Detector | R-AXIS IV$^{++}$ |
| Wavelength (Å) | 1.5418 |
| Resolution (Å) | 32.83–2.00 (2.11–2.00) |
| Unique reflections | 65505 (9450) |
| Multiplicity | 10.3 (9.6) |
| $\langle I/\sigma(I)\rangle$ | 20.6 (4.6) |
| Completeness (%) | 100 (100) |
| $R_{\rm merge}$† (%) | 8.6 (52.2) |

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the $i$th observation of reflection $hkl$ and $\langle I(hkl)\rangle$ is the average intensity of the $i$ observations.

### 2.5. Data collection and processing

For X-ray data collection, crystals were mounted on CryoLoops (Hampton Research), rinsed with cryoprotectant solution [30%(*v*/*v*) glycerol in reservoir solution] and flash-cooled directly in a nitrogen stream at 100 K. Diffraction data were collected with Cu $K\alpha$ radiation using a Rigaku FR-E+ SuperBright microfocus rotating-anode dual-wavelength (Cu and Cr) X-ray generator equipped with an R-AXIS IV$^{++}$ detector. Data sets were indexed, integrated and scaled using *MOSFLM* (Leslie, 1992) and the *CCP*4 suite (Winn *et al.*, 2011). Data-collection and processing statistics are given in Table 1.

## 3. Results and discussion

We successfully cloned and overexpressed a cylophilin A-like protein from *P. indica*. The overexpressed protein from 1 l bacterial cell culture in LB medium was subjected to initial purification by Ni–NTA His$_6$-affinity chromatography. The purified protein was digested with thrombin to remove the His tag. A benzamidine column was used to remove the thrombin. After thrombin digestion, PiCypA contained three additional residues, Gly-Ser-His, at the N-terminus. Further purification of PiCypA was carried out by size-exclusion chromatography on a HiLoad 16/60 Superdex 75 preparative-grade column

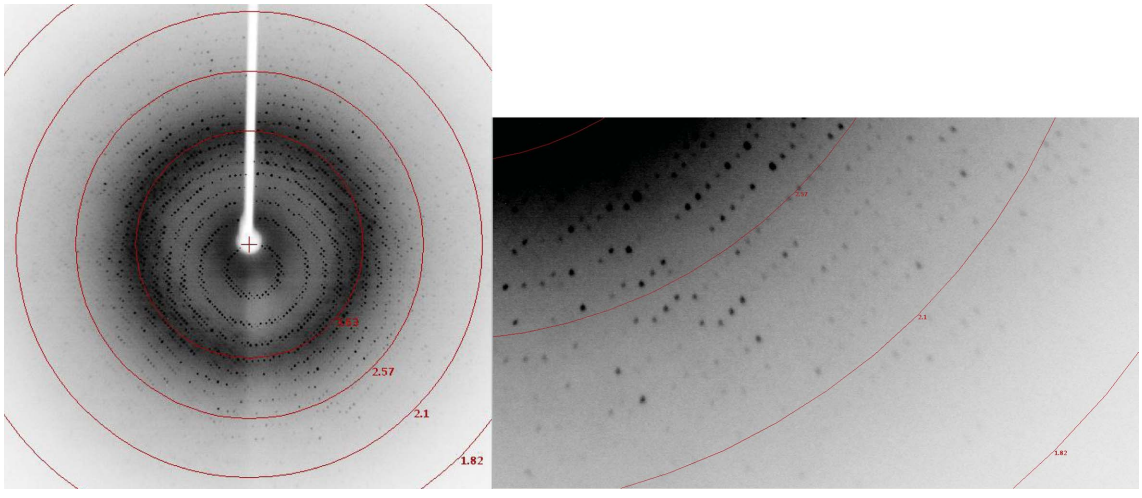



**Figure 3**
Representative X-ray diffraction image of an orthorhombic crystal of PiCypA. A small region of the image is enlarged for clarity. The red concentric circles show the resolution rings.

(GE Healthcare). The purified protein was >98% pure and gel-permeation chromatographic analysis revealed that it was a monomer in solution (Fig. 1). The total yield of purified protein was about 35 mg from 1 l culture. PiCypA crystallized in the orthorhombic space group $C222_1$, with unit-cell parameters $a$ = 121.15, $b$ = 144.12, $c$ = 110.63 Å (Fig. 2). Diffraction data were collected to 2.0 Å resolution (Fig. 3). Determination of the structure of PiCypA is in progress using the molecular-replacement method with the structural data for human cyclophilin A (PDB entry 3k0n; Fraser *et al.*, 2009), which shares 62% sequence identity with PiCypA, as a search model.

The diffraction data clearly indicated the presence of three molecules of PiCypA in the asymmetric unit, corresponding to a $V_M$ value of 4.48 Å$^3$ Da$^{-1}$ and a solvent content of ∼72.6% (Matthews, 1968). Interestingly, the packing views of PiCypA in the unit cell show a large solvent-accessible channel between tightly packed protein molecules when viewed down the $c$ axis. The molecular arrangements are very compact along the other two axes. Efforts are under way to solve the structure of PiCypA.

This study was supported by grants to NT and NSB from the Department of Science and Technology (DST), Government of India and ICGEB core funds. X-ray diffraction data were collected at the National Institute of Immunology, New Delhi. We thank Dr Amit Sharma for useful discussion and for the use of the crystallization facility. HB is the recipient of a Council for Scientific and Industrial Research (CSIR) senior research fellowship. DKT is the recipient of a Department of Biotechnology (DBT) junior research fellowship.

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