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Expression, purification, crystallization and preliminary X-ray diffraction analysis of *Arabidopsis thaliana* Deg8

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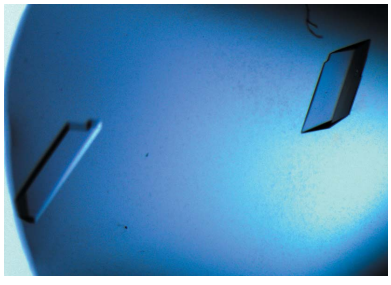
Arabidopsis thaliana Deg8, an ATP-independent serine endopeptidase, is involved in the repair of photosystem II (PSII), specifically the degradation of the photo-damaged PSII reaction centre D1 protein. To understand the molecular mechanism underlying the participation of Deg8 in the degradation of the photo-damaged D1 protein, the structure of Deg8 is needed. Until recently, however, no structure of Deg8 had been solved. In this study, Deg8 from *A. thaliana* was cloned, overexpressed and purified in *Escherichia coli*. Crystallization was performed at 277 K using tribasic sodium citrate as the precipitant and the crystals diffracted to 2.0 Å resolution, belonging to space group *C*2 with unit-cell parameters $a = 129.5$, $b = 124.2$, $c = 93.3$ Å, $\alpha = \gamma = 90$, $\beta = 132.4^\circ$. Assuming one trimer in the asymmetric unit, the Matthews coefficient and the solvent content were calculated to be 2.35 Å^3 Da^{-1} and 47.6%, respectively.

1. Introduction

Photo-inhibition is a phenomenon in which the activity of photosystem II (PSII) is inhibited when photosynthetic organisms are under stress from high light (Prásil *et al.*, 1992; Andersson & Aro, 2001). PSII is very sensitive to photo-inhibition and, among PSII proteins, PSII reaction centre D1 protein is the primary target for light-induced damage (Prásil *et al.*, 1992; Aro *et al.*, 1993; Sun *et al.*, 2007). The degradation of damaged D1 protein is very important for the dynamic equilibrium of PSII (Aro *et al.*, 1993; Andersson & Aro, 1997). During D1 protein degradation, primary cleavage appears to occur in the DE loop on the stromal side (Greenberg *et al.*, 1987; Cánovas & Barber, 1993; Shipton & Barber, 1994; Kanervo *et al.*, 1998) and in the AB and CD loops on the lumenal side (Barbato *et al.*, 1991; De Las Rivas *et al.*, 1992; Huesgen *et al.*, 2005).

The Deg proteases, which are ATP-independent serine endopeptidases, are present in both prokaryotes and eukaryotes (Huesgen *et al.*, 2005; Gottesman, 1996; Pallen & Wren, 1997; Adam & Clarke, 2002; Clausen *et al.*, 2002; Kieselbach & Funk, 2003). Deg proteases share a conserved structural feature of a trypsin-type protease domain in the N-terminal region with a conserved His–Asp–Ser catalytic triad, and between zero and three PDZ domains at the C-terminus (Pallen & Wren, 1997; Clausen *et al.*, 2002). DegP and DegS are two well studied Deg proteases in *Escherichia coli* with crystal structures solved in different oligomeric states. DegP plays an essential dual role as protease and molecular chaperone in the protein quality-control process, while DegS can sense unfolded outer-membrane proteins and initiate the σE-signalling proteolytic cascade (Wilken *et al.*, 2004; Krojer *et al.*, 2002, 2008; Sohn *et al.*, 2007; Jiang *et al.*, 2008; Shen *et al.*, 2009). In *Arabidopsis*, there are 16 genes coding for DegP-like proteases, named Deg1–16 (Huesgen *et al.*, 2005). Deg1, Deg5 and Deg8 were found in the lumen of the thylakoid, while Deg2 and Deg7 were found in the stroma (Huesgen *et al.*, 2005; Itzhaki *et al.*, 1998; Sun *et al.*, 2007, 2010). The locations of these Deg proteases and their potential serine protease activity give a hint that they may be responsible for the degradation of PSII reaction centre D1 protein. Deg1 has been well studied and found to be able to degrade the photo-damaged D1 protein of PSII (Kapri-Pardes *et al.*, 2007). Deg8, which was predicted to comprise a protease domain in the N-terminal region and only one PDZ domain in the C-terminal



region, has the same domain structure as Deg1. A recent study has shown that Deg8 has proteolytic activity toward both β-casein and PSII reaction centre D1 protein (Sun *et al.*, 2007). An *Arabidopsis* line in which T-DNA was inserted into Deg8 showed increased sensitivity to photo-inhibition and degradation of D1 protein was slowed in the mutant under high irradiance (Sun *et al.*, 2007; Kato *et al.*, 2012).

Here, we report the crystallization of *Arabidopsis* Deg8 S292A, a proteolytically inactive mutant. The three-dimensional structure, which is being determined by molecular replacement, will provide a molecular insight into the participation of Deg8 in the degradation of the photo-damaged D1 protein.

2. Materials and methods

2.1. Cloning and expression of Deg8

E. coli strain BL21 (DE3), which contains a plasmid (pET28a, Novagen) carrying the gene fragment of a 358 amino-acid sequence of wild-type *Arabidopsis thaliana* Deg8 with its signal peptide (1–90 amino acids) deleted, has been described in detail previously (Sun *et al.*, 2007). Deg8 S292A, a proteolytically inactive Deg8 mutant with the serine of the catalytic triad exchanged for alanine, was made by polymerase chain reaction (PCR) using a QuikChange Site-Directed Mutagenesis Kit (Stratagene), and the PCR product (plasmid) was verified by direct DNA sequencing. Deg8 S292A is hereafter referred to as His-Deg8-His S292A since it has His tags at its N-terminal and C-terminal ends. Another form, Deg8-His S292A with only one His tag at the C-terminal end, was also constructed by using the primers DEG8-C-forward, CATGCCATGGGCCTTGGTGATCCATCCG-TTGCAAC, and DEG8-C-reverse, CCGCTCGAGTGAACTTTTC-TCTTCCAATGAGATCTTTAACTC.

2.2. Purification of Deg8

E. coli strain BL21 (DE3) cells transformed with expression plasmid were grown in 0.8 l Luria–Bertani (LB) medium containing 25 µg ml^{-1} kanamycin at 310 K until the OD_{600} reached 0.8. Expression of recombinant protein was induced by the addition of IPTG to a final concentration of 0.5 m*M* and growth of the cells was continued for 12 h at 289 K. The cells were harvested by centrifugation at 4000*g* for 30 min at 277 K, suspended in 40 ml buffer *A* (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl) and lysed by sonication. The lysate was centrifuged at 40 000*g* for 60 min. The supernatant obtained was loaded onto a 3 ml GE Chelating Sepharose Fast Flow column (pre-equilibrated with buffer *A*), rinsed with 40 ml buffer *B* (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 20 m*M* imidazole) and then with buffer *C* (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 50 m*M* imidazole) and finally eluted with 20 ml buffer *D* (50 m*M* Tris–HCl pH 7.5, 150 m*M* NaCl, 200 m*M* imidazole). For further purification, gel filtration (with buffer *E*, 50 m*M* MES pH 6.0, 150 m*M* NaCl) was performed on an ÄKTA FPLC (GE Healthcare) on Superdex 200 10/300 GL columns (GE Healthcare) at a flow rate of 0.5 ml min^{-1} at 277 K. The fractions containing the protein peak were collected and assayed by SDS–PAGE. SDS–PAGE was performed with 12%(*w*/*v*) polyacrylamide gels and the gels were stained with Coomassie Brilliant Blue R-250. For crystallization, the purified protein was concentrated to approximately 12 mg ml^{-1} using a Millipore Amicon Ultra Centrifugal Filter (10 kDa).

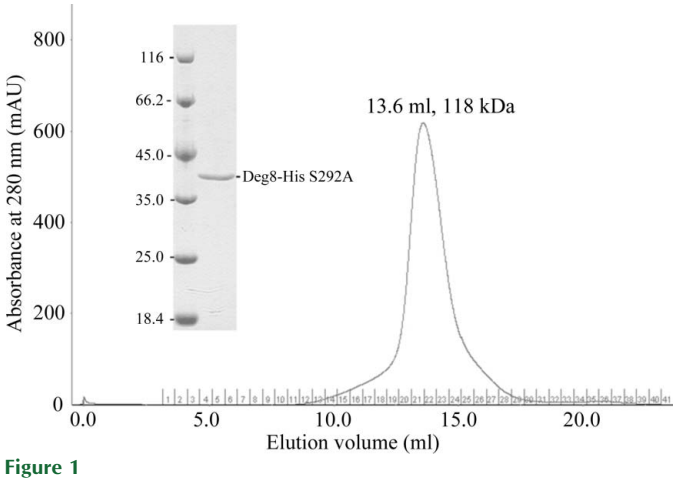


Figure 1
Purification of Deg8-His S292A by Superdex 200 10/300 GL gel filtration: gel-filtration profile of the recombinant Deg8-His S292A and reducing SDS–PAGE gel (12%) of the corresponding purified protein (the left lane contains molecular markers labelled in kDa).

2.3. Crystallization of Deg8

Crystallization screening was carried out using the hanging-drop method at 277 and 289 K. Initial crystallization trials were performed using the commercial crystallization reagent kits Crystal Screen, Crystal Screen 2 and Index (Hampton Research). 1 µl protein solution (at a concentration of 6 or 12 mg ml^{-1}) was manually mixed with 1 µl reservoir solution and equilibrated against 100 µl reservoir solution. For Deg8-His S292A, crystals were found to appear in 3 d at both 277 and 289 K from several conditions in the Index kit such as condition Nos. 40 [0.1 *M* citric acid pH 3.5, 25%(*w*/*v*) polyethylene glycol 3350], 41 [0.1 *M* sodium acetate trihydrate pH 4.5, 25%(*w*/*v*) polyethylene glycol 3350], 43 [0.1 *M* bis-Tris pH 6.5, 25%(*w*/*v*) polyethylene glycol 3350], 46 [0.1 *M* bis-Tris pH 6.5, 20%(*w*/*v*) polyethylene glycol monomethyl ether 5000], 79 [0.2 *M* ammonium acetate, 0.1 *M* bis-Tris pH 6.5, 25%(*w*/*v*) polyethylene glycol 3350], 80 [0.2 *M* ammonium acetate, 0.1 *M* HEPES pH 7.5, 25%(*w*/*v*) polyethylene glycol 3350], 81 [0.2 *M* ammonium acetate, 0.1 *M* Tris pH 8.5, 25%(*w*/*v*) polyethylene glycol 3350] and 94 [0.2 *M* sodium citrate tribasic dihydrate, 20%(*w*/*v*) polyethylene glycol 3350], but none from Crystal Screen and Crystal Screen 2. Further optimization was conducted for conditions yielding crystals by variation of protein concentration, precipitant gradient and the pH of the buffer and by the use of additives (Hampton Research). Finally, crystals suitable for data collection were obtained within 1 d at 277 K from the following improved condition based on condition 94 of the Index kit: 0.2 *M* sodium citrate tribasic dihydrate, 16.25% PEG 3350, 5% Jeffamine M600. For Deg8-His, His-Deg8, His-Deg8-His and His-Deg8-His S292A, no crystals suitable for data collection were obtained.

2.4. Data collection and X-ray diffraction analysis

Diffraction data were collected from Deg8-His S292A crystals at a wavelength of 0.9788 Å at 100 K on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF) equipped with a MAR CCD 225 detector. 10% MPD was used as a cryoprotectant for data collection under cryogenic conditions. The crystals were soaked for several seconds in reservoir solution containing the cryoprotectant and then flash-cooled directly in liquid nitrogen. A complete data set was collected to 2.0 Å. The oscillation angle was 1°. The diffraction data were indexed, integrated and scaled with *DENZO* and the *HKL-2000* software package (Otwinowski & Minor, 1997).

Table 1
X-ray diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	Synchrotron radiation, SSRF
Wavelength (Å)	0.9787
Detector	MAR 225
Space group	*C*2
Resolution (Å)	50–2.0 (2.07–2.00)
Unit-cell parameters (Å, °)	a = 129.5, b = 124.2, c = 93.3, $\alpha = \gamma = 90$, $\beta = 132.4$
Total reflections	546280
Unique reflections	73748
Completeness (%)	99.7 (100)
$R_{\rm merge}$† (%)	6.8 (47.8)
$\langle I/\sigma(I)\rangle$	25.2 (4.3)
Multiplicity	7.4 (7.7)

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

3. Results and discussion

Recombinant Deg8 (residues 91–448, excluding the signal peptide) from *A. thaliana* (from five constructs: Deg8-His, His-Deg8, His-deg8-His, His-Deg8-His S292A and Deg8-His S292A) was expressed in *E. coli* BL21 (DE3) and purified to homogeneity by two chromatographic steps, Ni-affinity chromatography and gel filtration, resulting in a yield of approximately 30 mg purified protein from 0.8 l culture. SDS–PAGE analysis of the purified Deg8 showed a single band corresponding to a molecular weight of about 42 kDa (His-Deg8-His S292A, not shown) or about 39 kDa (Deg8-His S292A; Fig. 1), which is close to the calculated molecular weight of Deg8 with the respective His tag(s) (41.49 or 38.58 kDa). The gel-filtration chromatography profiles from a Superdex 200 column eluted with 150 m*M* NaCl showed single peaks, and the molecular weights estimated in accordance with the standard proteins correspond to the expected trimer (∼120 kDa for Deg8-His S292A; Fig. 1). The buffer pH for gel filtration was selected to be ∼6.0, which is consistent with the fact that the lumenal side of the thylakoid, the natural environment for Deg8, appears to be weakly acidic.

The crystals showing the best diffraction were obtained with Deg8-His S292A (Fig. 2). A summary of the data-processing statistics is shown in Table 1. The crystal belonged to space group *C*2, with unit-cell parameters a = 129.5, b = 124.2, c = 93.3 Å, $\alpha = \gamma = 90$, $\beta = 132.4°$. It is worth noting that the cryoprotectant was critical for Deg8 crystal diffraction. We screened different reagents such as PEG 400, PEG 4000, MPD and glycerol at different concentrations. The crystals without cryoprotectant or soaked in solutions with PEG or glycerol diffracted to only 4 Å resolution. However, MPD dramatically improved the diffraction, extending the resolution limit of Deg8 crystals to 2 Å (Fig. 3).

In the crystal structures of Deg proteins reported to date, three monomers form a trimer as a basic structural unit. The gel-filtration experiment showed that *A. thaliana* Deg8 also exists as a trimer in solution. Assuming one trimer per asymmetric unit, the Matthews coefficient (Matthews, 1968) ($V_{\rm M}$) is 2.35 Å^3 Da^{-1}, corresponding to a solvent content of 47.6%. The self-rotation function calculation also

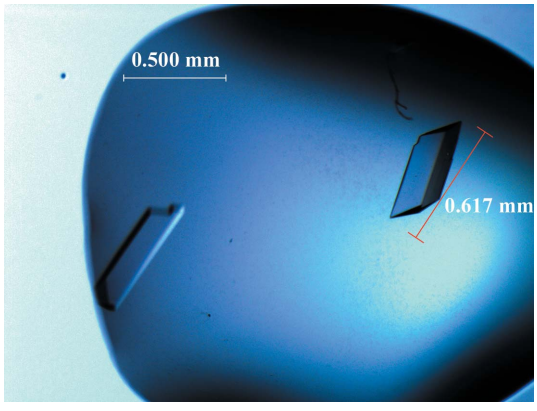


Figure 2
Typical crystal of Deg8-His S292A.

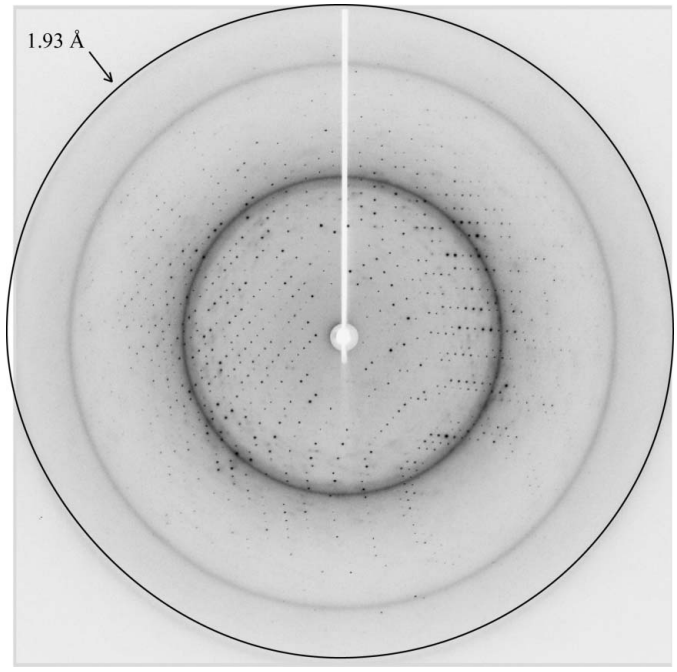


Figure 3
X-ray diffraction image of Deg8-His S292A crystal.

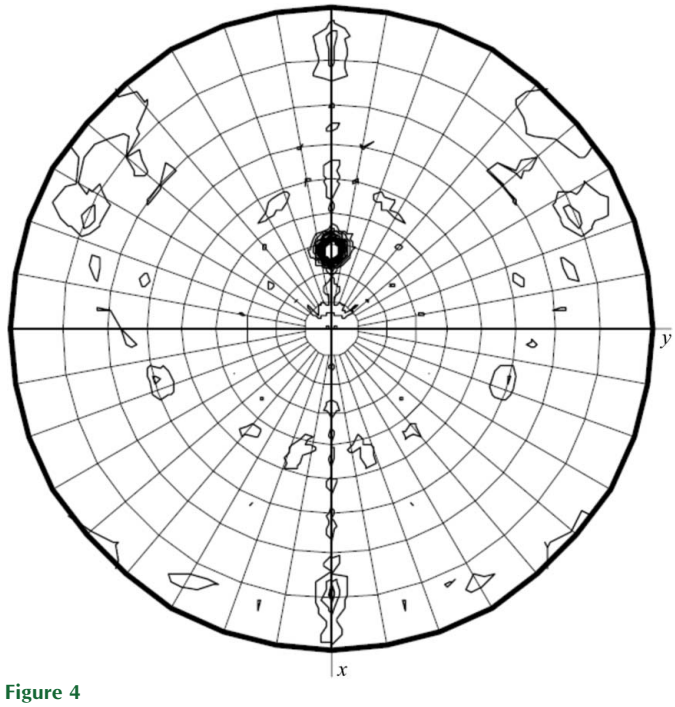


Figure 4
Self-rotation function calculation map (χ = 120.0°).

showed the presence of a noncrystallographic (NCS) threefold axis in the $\chi = 120.0^{\circ}$ section (Fig. 4).

Crystals of His-Deg8-His S292A were poor in appearance, diffraction ability and reproducibility; none were suitable for data collection. Interestingly, most Deg protease constructs that have been successful in both crystallization and data collection [*e.g.* DegP, DegS from *E. coli* (Krojer *et al.*, 2002; Sohn *et al.*, 2007; Shen *et al.*, 2009; Wilken *et al.*, 2004) and Deg1 from *A. thaliana*, crystallized in our research group] have only one His tag at their C-terminal end, the sole exception being Deg5 crystallized with His tags at both the N- and C-terminal ends.

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