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

Purification, crystallization and X-ray diffraction study of extracellular dermal glycoprotein from carrot and the inhibition complex that it forms with an endo-β-glucanase from *Aspergillus aculeatus*

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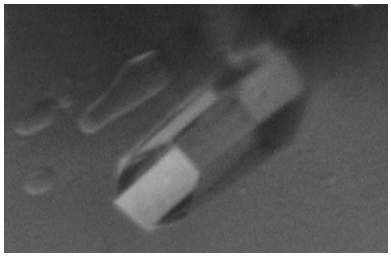
Received 7 April 2011
Accepted 26 May 2011



Extracellular dermal glycoprotein (EDGP) may play an important role in the plant defence system of the carrot (*Daucus carota*) as it has inhibitory activity against endo-β-glucanase produced by invading pathogens. Here, EDGP and the inhibition complex that it forms with FI-CMCase, a carboxyl methyl cellulase from *Aspergillus aculeatus*, were successfully crystallized. The hexagonal crystal of EDGP belonged to space group $P6_2$, with unit-cell parameters $a = b = 130.4$, $c = 44.5$ Å, $\gamma = 120^\circ$. The monoclinic crystal of the complex of EDGP with FI-CMCase belonged to space group $C2$, with unit-cell parameters $a = 169.5$, $b = 143.0$, $c = 63.0$ Å, $\beta = 110.9^\circ$.

1. Introduction

Plant cell walls are composed of various polysaccharides such as cellulose, hemicellulose and pectin. Cellulose microfibrils are linked *via* hemicellulose. This cellulose–hemicellulose network provides tensile strength and acts as a physical barrier against invading pathogens. To penetrate and use plant cell walls nutritionally, pathogens secrete cell-wall-degrading enzymes. These enzymes, which include endoglucanases, xylanases and polygalacturonases, are classified into glycoside hydrolase (GH) families in the CAZY database (Henrissat, 1991). In response to pathogenic attack, plants produce proteinous inhibitors against these cell-wall-degrading enzymes (Juge, 2006; Lagaert *et al.*, 2009). Extracellular dermal glycoprotein (EDGP) is one such proteinous inhibitor against these degrading enzymes. EDGP shows inhibitory activity towards the xyloglucan-specific endo-β-1,4-glucanase (XEG) from *Aspergillus aculeatus* (Shang *et al.*, 2005), which belongs to the GH12 family and specifically cleaves xyloglucan, which is a major hemicellulose found in dicots (Pauly *et al.*, 1999). Xyloglucan consists of a β-linked glucose backbone substituted with xylose side chains. The degradation of xyloglucan causes great damage to dicotyledonous plants. Consequently, the inhibition of XEG by EDGP is an important component of the defence system of the carrot.

Homologous proteins to EDGP are widely present in plants and several of these homologous proteins have been characterized. Tomato (*Lycopersicon esculentum*) XEGIP (xyloglucan-specific endo-β-1,4-glucanase inhibitor protein) inhibits XEG by forming an associated 1:1 complex (Qin *et al.*, 2003). Necturin IV (NEC IV) from tobacco (*Nicotiana langsdorffii* × *N. sanderae* var. LxS8) also inhibits XEG (Naqvi *et al.*, 2005; Sansen *et al.*, 2004). In contrast, the homologous protein from wheat (*Triticum aestivum* xylanase inhibitor; TAXI) inhibits GH11 xylanases (Gebruers *et al.*, 2004; Sansen *et al.*, 2004). Interestingly, the soybean (*Glycine max* L. Merrill cv. Miyagishirome) homologue basic 7S globulin (Bg7S) lacks inhibitory activity for both GH11 and GH12 enzymes (Yoshizawa *et al.*, 2011). EDGP shares 61, 62, 21 and 37% amino-acid identity with tomato XEGIP, tobacco NEC IV, wheat TAXI-IA and soybean Bg7S, respectively. Structural analyses of TAXI and Bg7S have already been reported (Sansen *et al.*, 2004; Yoshizawa *et al.*, 2011). However, structures have not been determined for EDGP and homologous proteins that inhibit GH12 enzymes.

Expression of EDGP is induced by both biotic and abiotic stress (Satoh *et al.*, 1986). EDGP consists of 413 amino-acid residues and is

subject to post-translational modifications; the processed protein contains N-terminal pyroglutamic acid, six disulfide-bond pairs and four N-linked glycosylation sites (Shang *et al.*, 2004). Deglycosylation of EDGP causes a complete loss of its ability to inhibit XEG (Shang *et al.*, 2005). To clarify the inhibition mechanism of EDGP towards GH12 endo-β-glucanase, we used X-ray crystallography to determine the structure of both EDGP and EDGP in complex with a GH12 enzyme. In the present paper, we describe the purification and crystallization of EDGP and EDGP in complex with FI-CMCase, a GH12 endo-β-glucanase from *A. aculeatus* (Kanda *et al.*, 1976), and also detail the initial diffraction analysis of these crystals.

2. Materials and results

2.1. Preparations of EDGP and FI-CMCase for crystallographic study

EDGP was purified from carrot callus culture medium. Carrot callus was grown for 2–3 weeks at 298 K in Murashige–Skoog (MS) medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid. The carrot callus culture suspension was filtered using Miracloth (Merck KGaA) and clarified by centrifugation for 30 min at 277 K (43 667*g*). An equal volume of 50 m*M* sodium acetate pH 4.6 was added to the supernatant and it was applied onto a HiTrap SP HP column (GE Healthcare) equilibrated with 50 m*M* sodium acetate pH 4.6. The bound proteins were eluted with a linear gradient from 0 to 400 m*M* NaCl. Fractions containing EDGP were concentrated to 20 mg ml^{-1} in 20 m*M* potassium phosphate pH 7.4 using Amicon Ultra 30 kDa molecular-weight cutoff filter units (Millipore). The purity of EDGP was confirmed by SDS–PAGE with Coomassie Brilliant Blue (CBB) staining (Fig. 1, lane 1). About 1 mg purified EDGP was obtained from 400 ml culture.

The cDNAs encoding FI-CMCase were obtained by PCR-based gene synthesis and inserted into pGEX6P-I vector (GE Healthcare) at the *Bam*HI–*Xho*I site. The plasmid encodes FI-CMCase with a GST tag at the N-terminus. The expression vector was introduced into *Escherichia coli* strain BL21. The cells were grown at 310 K to an optical density of 0.6 at 660 nm in LB medium containing 50 µg ml^{-1} ampicillin. After the addition of 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG), cells were grown for 6 h at 298 K. The cells were then harvested, suspended in lysis buffer (50 m*M* HEPES–NaOH pH 7.4, 250 m*M* NaCl, 5 m*M* EDTA and 0.5 m*M* PMSF) and disrupted by sonication. After centrifugation, the supernatant was applied onto glutathione Sepharose 4B (GS4B) resin (GE Healthcare). The bound proteins were eluted with 50 m*M* Tris–HCl pH 9.0, 200 m*M* NaCl and 30 m*M* reduced glutathione. Fractions containing the GST-fused FI-CMCase were dialyzed with 50 m*M* Tris–HCl pH 9.0 and 200 m*M* NaCl. The N-terminal GST tag was cleaved by HRV3C protease at 277 K for 15 h and the solution was then applied onto GS4B resin to remove GST. The flowthrough fractions containing FI-CMCase were applied onto a HiLoad Superdex 75 column (GE Healthcare) equilibrated with 5 m*M* Tris–HCl pH 9.0 and 100 m*M* NaCl. Fractions containing FI-CMCase were concentrated to 20 mg ml^{-1} using Amicon Ultra 10 kDa molecular-weight cutoff filter units (Millipore). The purity of FI-CMCase was confirmed by SDS–PAGE with CBB stain (Fig. 1, lane 2). The EDGP–FI-CMCase complex was prepared by mixing EDGP and FI-CMCase in an equimolar ratio (13 mg ml^{-1} EDGP and 7 mg ml^{-1} FI-CMCase in 13 m*M* potassium phosphate pH 7.4, 2 m*M* Tris–HCl pH 9.0 and 35 m*M* NaCl).

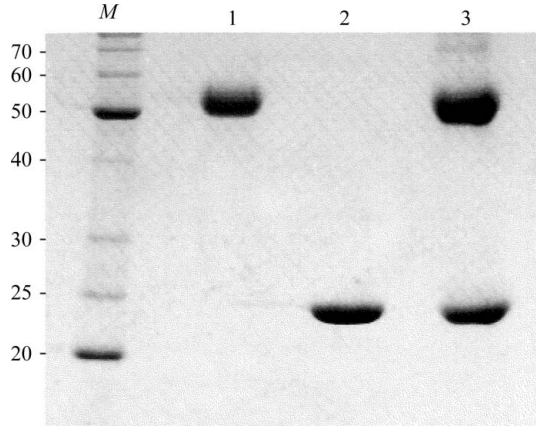


Figure 1
SDS–PAGE analysis of purified EDGP (lane 1), purified FI-CMCase (lane 2) and a crystal of the EDGP–FI-CMCase complex (lane 3). Lane *M* contains a molecular-size marker (labelled in kDa).

2.2. Crystallization and initial crystallographic study of EDGP and the EDGP–FI-CMCase complex

Screening of crystallization conditions for EDGP and the EDGP–FI-CMCase complex was performed by the sitting-drop vapour-diffusion method using a Hydra II Plus One (Matrix) and more than

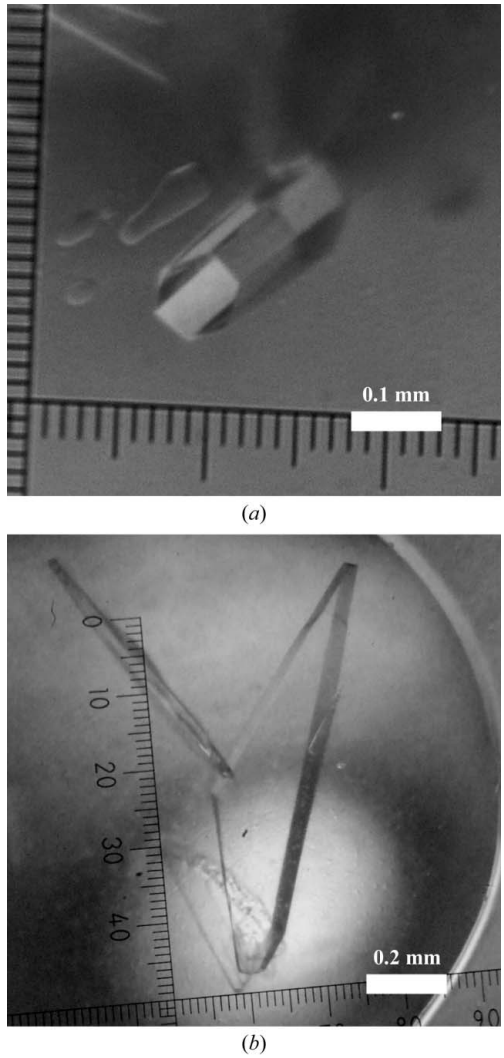


Figure 2
Crystals of EDGP (*a*) and the EDGP–FI-CMCase complex (*b*).

Table 1
Data-collection statistics for crystals of EDGP and the EDGP–FI-CMCase complex.

Values in parentheses are for the highest resolution shell.

		EDGP (Pt derivative)				
	EDGP (Native)	Peak	Edge	High remote	Low remote	EDGP–FI-CMCase
Wavelength (Å)	0.9000	1.07244	1.07171	1.05420	1.07710	1.0000
Resolution range (Å)	50.0–1.00 (1.04–1.00)	50.0–1.80 (1.86–1.80)	50.0–1.80 (1.86–1.80)	50.0–1.80 (1.86–1.80)	50.0–1.80 (1.86–1.80)	50.0–2.50 (2.59–2.50)
Measured reflections	843817 (41436)	439831 (40733)	441642 (37085)	427386 (25357)	409988 (15200)	185159 (18464)
Unique reflections	223206 (19344)	40397 (4033)	40518 (4031)	40545 (3962)	40315 (3619)	48804 (4859)
Multiplicity	3.8 (2.2)	10.9 (10.1)	10.9 (9.2)	10.5 (6.4)	10.2 (4.2)	3.8 (3.8)
Completeness (%)	96.2 (83.5)	100.0 (100.0)	100.0 (100.0)	99.9 (98.8)	98.9 (89.4)	99.9 (100.0)
Mean $I/\sigma(I)$	10.5 (1.5)	16.5 (6.4)	15.5 (5.0)	14.1 (3.1)	13.2 (1.9)	14.4 (5.5)
$R_{\rm merge}$† (%)	6.0 (37.0)	8.3 (30.6)	8.1 (35.4)	8.6 (44.0)	8.6 (52.6)	7.1 (30.2)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

480 different reservoir solutions from commercially available screening kits (Hampton Research, Molecular Dimensions and Qiagen) at 293 K. Crystals of EDGP were obtained using the following reservoir solutions: No. 10 from Crystal Screen (0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 4.6 and 30% PEG 4000), No. 82 from the JCSG+ suite (0.15 *M* potassium bromide and 30% PEG MME 2000) and No. 94 from the JCSG+ suite (0.2 *M* ammonium acetate, 0.1 *M* Bis-Tris pH 5.5 and 25% PEG 3350). The buffer, the pH and the salt and precipitant concentrations of these crystallization conditions were optimized using the hanging-drop vapour-diffusion method. Hexagonal crystals of EDGP suitable for diffraction studies were obtained using a reservoir solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 4.6 and 30% PEG MME 2000 after several days (Fig. 2*a*). Prior to the X-ray experiment, the EDGP crystal was transferred into a cryoprotectant solution consisting of 0.2 *M* ammonium acetate, 0.1 *M* sodium acetate pH 4.6 and 40% PEG MME 2000 with a nylon loop and then cooled in an N_2-gas stream at 100 K. X-ray diffraction data for the EDGP crystal were collected using a Quantum 315 CCD detector (Area Detector Systems Corp.) on beamline BL-41XU at SPring-8 (Sayo, Japan). Diffraction data were integrated, scaled and averaged with the *HKL*-2000 program suite (Otwinowski & Minor, 1997). The EDGP crystal belonged to space group $P6_2$, with unit-cell parameters $a = b = 130.4$, $c = 44.5$ Å, $\gamma = 120^\circ$. Data-collection statistics are summarized in Table 1. The asymmetric unit of the EDGP crystal is estimated to contain a single molecule ($V_{\rm M} = 2.43$ Å^3 Da^{-1}). EDGP contains 12 cysteines and six disulfide-bond pairs. Therefore, a heavy-atom derivative was prepared by soaking the native crystal in reservoir solution containing 10 m*M* K_2PtCl_4 for 15 h. X-ray diffraction data for the platinum-derivative crystal were collected using a Quantum 270 CCD detector (Area Detector Systems Corp.) on beamline BL-17A at Photon Factory (PF). The wavelengths for MAD measurements were selected based on XAFS measurements. Crystals of the EDGP–FI-CMCase complex were obtained by initial screening using the following reservoir solutions: No. 89 from the PACT suite (0.2 *M* sodium nitrate, 0.1 *M* Bis-Tris propane pH 8.5 and 20% PEG 3350) and No. 9 from the Protein Complex suite (0.2 *M* sodium chloride, 0.1 *M* MES pH 6.0 and 20% PEG MME 2000). After these crystallization conditions had been optimized, a monoclinic crystal of the EDGP–FI-CMCase complex suitable for diffraction study was obtained using a reservoir solution consisting of 0.4 *M* sodium chloride, 0.1 *M* MES pH 6.0, 22% PEG MME 2000 and 5% glycerol (Fig. 2*b*) after 10 d. The crystal was confirmed to contain EDGP and FI-CMC by SDS–PAGE using CBB stain (Fig. 1, lane 3). For cryoprotection, the EDGP–FI-CMCase complex crystal was transferred into a cryoprotectant solution consisting of 0.4 *M* sodium chloride, 0.1 *M* MES pH 6.0, 22% PEG MME 2000 and 20% glycerol. X-ray diffraction data for the EDGP–FI-CMCase complex crystal were collected using a Quantum 270 CCD detector (Area Detector Systems Corp.) on the NE-3A beamline at Photon Factory (PF). The crystal belonged to space group *C*2, with unit-cell parameters $a = 169.5$, $b = 143.0$, $c = 63.0$ Å, $\beta = 110.9^\circ$. The asymmetric unit was estimated to contain two EDGP–FI-CMCase complexes ($V_{\rm M} = 2.58$ Å^3 Da^{-1}). Structure determination of EDGP and the EDGP–FI-CMCase complex is now in progress.

We acknowledge the kind support of the beamline staff at SPring-8 and PF. This work was supported by grants from KAKENHI, the National Project on Protein Structural and Functional Analyses (Protein 3000 Project) and the Targeted Proteins Research Program to MS, TS and HH from MEXT. We thank Dr H. Shiota, Yokohama City University for supplying carrot callus and Dr C. Everroad, Riken Yokohama Institute for English corrections.

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