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Purification, crystallization and preliminary X-ray diffraction analysis of a novel mannose-binding lectin from *Gastrodia elata* with antifungal properties

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A plant antifungal protein, *Gastrodia* antifungal protein (GAFP-1) has been isolated from terminal corms of the orchid *Gastrodia elata* B1 f. *elata*, purified to homogeneity and crystallized by means of the hanging-drop vapour-diffusion method. The best quality crystals grew over several months at 277 K. The crystal used for data collection belongs to the space group $P2_12_12$, with unit-cell parameters a = 61.087, b = 91.488, c = 81.132 Å. Using a synchrotron-radiation source, the resolution limit of the data reached 2.0 Å, with an overall $R_{\rm merge}$ of 0.097 and a completeness of 99.8%. Four independent molecules were estimated to be present in the asymmetric unit, with a solvent content of 46.3%. This data will help to solve the first structure of a monomeric monocot mannose-binding lectin.

1. Introduction

Lectins are a structurally diverse group of proteins widely distributed in almost all types of living organisms. One of the four main families of plant lectins are the monocot mannose-binding lectins, which have been extensively studied in the last decade (Barre *et al.*, 2001). A novel class of antifungal proteins belonging to this family, known as the gastrodianin-like proteins (GLIPs), have recently been found in the orchid *Gastrodia elata* (Wang *et al.*, 2001).

For thousands of years, the orchid *G. elata* has been cultured as a traditional Chinese medicinal herb. This plant lacks chlorophyll and leads a parasitic life on the fungus *Armillaria mellea*. The fungal hyphae invade the nutritive and primary corms of *G. elata* during its development. The cortical cells in corms, however, arrest and digest the infecting hyphae and transport the released nutrients into a terminal corm for sustaining its growth (Yang & Hu, 1990). Interestingly, the fungi can never invade the terminal corm. The first protein showing resistance against fungal infection was isolated from the cortex of terminal corms (Hu *et al.*, 1988) and was named *Gastrodia* antifungal protein (GAFP). It is also known as gastrodianin. Histochemical localization *in vivo* using the immunofluorescence method showed that the quantity of GAFP increased 15 times in nutritive corms on infection by fungi. *In vitro* assays also showed the strong inhibitive activity of GAFP towards the progression of hyphal growth of *Trichoderma viride* (Hu & Huang, 1994). Further tests indicated that GAFP has a wide-range antifungal activity upon phytopathogenic fungi such as *Valsa ambiens*, *Rhizoctonia solani*, *Gibberella zeae*, *Ganoderma lucidum* and *Botrytis cinerea* (Xu *et al.*, 1998). Unlike some other monocot mannose-binding lectins, GAFP shows no activity to agglutinate either human B type erythrocytes or trypsin-treated erythrocytes, but is nevertheless a lectin as defined by Peumans & Van Damme (1995) since GAFP can reversibly bind to mannose, *N*-acetylglucosamine and chitin (Xu *et al.*, 1998). Several cDNAs encoding GAFP have been cloned and sequenced by Hu *et al.* (1999) and Wang *et al.* (1999, 2001) separately, with slight sequence differences between them. The amino-acid sequence of GAFP-1, the most active of these isoforms, was determined by automatic Edman degradation, matching the nucleotide sequence quite well (Hu *et al.*, 1999). Searching for homologous sequences in databanks using the *BLAST* program (NCBI) revealed that GAFP-1 shared a certain degree of homology with subunits of some monocot mannose-binding lectins, for instance the lectins from the orchids *Epipactis helleborine* and *Listera ovata* (Van Damme *et al.*, 1994), snowdrop (*Galanthus nivalis*) (GNA; Van Damme *et al.*, 1991), garlic (*Allium sativum*; ASAI) (Chandra *et al.*, 1999) and *Narcissus pseudonarcissus* (NPL; Sauerborn *et al.*, 1999) (Fig. 1). Nevertheless, the monomeric form of GAFP has been biochemically confirmed, whereas most proteins of this family are functionally dimeric or tetrameric and display no

antifungal activity (Wang *et al.*, 2001). Another noticeable fact is that the sequence of GAFP-1 shows no similarity to those of any known antifungal protein classes. Thus, it is possible that GAFP inhibits fungal growth with a novel mechanism completely different from those reported previously. Therefore, determining the three-dimensional structure of GAFP-1 becomes more intriguing in order to understand the details of its antifungal properties. To date, no crystal structure of a functionally monomeric monocot mannose-binding lectin has been reported, despite there being several available structures of multimeric lectins such as GNA (Hester *et al.*, 1995), NPL (Sauerborn *et al.*, 1999), garlic lectin (Chandra *et al.*, 1999) and *Scilla campanulata* agglutinin (SCA; Wood *et al.*, 1999). In this context, we report here the purification, crystallization and preliminary X-ray diffraction analysis of GAFP-1. The results of this work should enable us to solve the three-dimensional structure of this novel type of antifungal protein.

2. Methods and results

2.1. Purification

Newborn terminal corms of the orchid *G. elata* B1 f. *elata* were collected from a local garden culture, each of which weighed 10–80 g. Approximately 1.0 kg of corms were chopped up and homogenized in 0.2 *M* sodium chloride pre-chilled at 277 K, followed by centrifugation at 10 000*g* for 10 min at the same temperature. Solid ammonium sulfate was added to the supernatant to 80% saturation. After 20 min incubation on ice, the precipitate was dissolved in distilled water. The total protein extract was obtained after sufficient dialysis against distilled water and the removal of insoluble precipitates by centrifugation. GAFP-1 was purified using chromatography steps as follows. The sample was firstly loaded onto a DEAE-Sephadex column to remove pigments and acidic impurities. The flowthrough fraction was concentrated to the desired degree by means of ultrafiltration before application to a Sephadex column pre-equilibrated with 0.05 *M* phosphate buffer pH 6.0. The elution peak showing obvious antifungal activity was pooled and the pH was adjusted to 6.5, after which the protein was absorbed by a CM-cellulose column at the same pH and developed with a linear gradient of 0–0.3 *M* sodium chloride. The eluent with the highest antifungal capability of the eluted peaks was collected and then desalted and concentrated by ultrafiltration with PM5 membranes. The purified protein was stored as a lyophilized powder. The overall yield of GAFP-1 was roughly 20 mg from 1000 g of fresh corms, as estimated by scanning of the SDS–PAGE gel. The pI value of GAFP-1 is about 8.3, determined by IEF using polyacrylamide gels containing 3% ampholine with pH range 3–9.5. The exact molecular mass of GAFP-1, as measured by MALDI-TOF mass spectrometry, is 12 296.30 Da (Fig. 2). This value is in good agreement with the theoretical mass calculated from its primary structure reported by Hu *et al.* (1999), assuming that the mature peptide corresponds to the partial sequence from residues 1 to 112 (Fig. 1). This result clearly indicates that the purified protein consists of 112 amino-acid residues, rather than 129 residues as reported previously (Hu *et al.*, 1999).

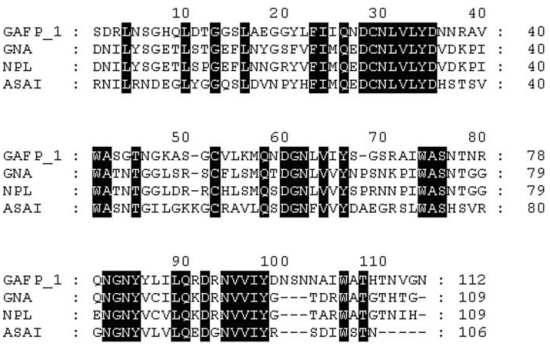

Figure 1
Amino-acid sequence alignment of GAFP-1 with the subunit sequences of some multimeric monocot mannose-binding lectins from snowdrop (*Galanthus nivalis*) (GNA), *Narcissus pseudonarcissus* (NPL) and garlic (*Allium sativum*) (ASAI). Identities among these sequences are highlighted.

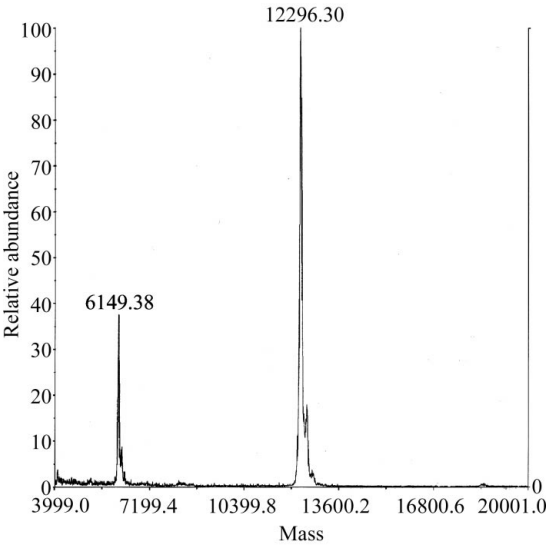

Figure 2
MALDI-TOF mass spectrum of GAFP-1

2.2. Assay of antifungal activity

The inhibitory activity of GAFP-1 against fungi was tested on 9 cm Petri dishes. 5 µl of sterilized protein solutions of concentrations from 0.5 to 2.0 mg ml^{-1} in 0.05 *M* phosphate buffer pH 6.5 were added to the surface of PDA medium when fungal inoculums grow to a diameter of 3 cm at 278 K. The same amount of buffer was also added simultaneously as a control. After incubation for 6–24 h, the length of the hyphae was measured and recorded. To quantify the antifungal ability of GAFP-1, $IC_{0.5}$ is defined as the concentration of the protein solution required to retard the hyphal growth by 50% of the normal length compared with that of the control. In our experiment, the strong inhibitory activity of GAFP-1 against *Trichoderma viride* was expressed in its $IC_{0.5}$ value of 0.08 mg ml^{-1}.

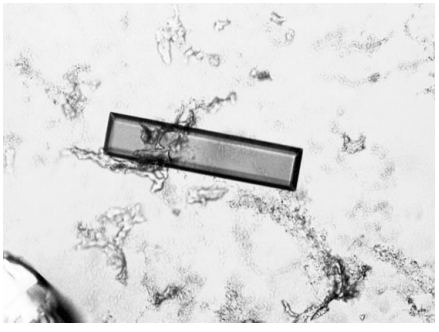

Figure 3
The best crystal of GAFP-1 used for data collection (0.2 × 0.02 × 0.04 mm).

2.3. Crystallization

Diffraction-quality crystals were grown at 277 K by means of the hanging-drop vapour-diffusion method (Ducruix & Giegé, 1992). The lyophilized protein was dissolved in 0.02 *M* acetic acid to a final concentration of 10 mg ml^{-1} as a stock solution. The precipitant was composed of 1.8 *M* ammonium sulfate in 0.1 *M* MES buffer pH 6.5; 2.5% dioxane and 0.1 *M* sodium potassium tartrate were used as additives. Drops were produced by mixing equal volumes of protein solution and precipitant solution,

while 0.4–0.8 ml of reservoir was added to the wells. Crystals of GAFP-1 grew so slowly that crystal nuclei could only just be observed after at least one month. Obtaining crystals large enough for X-ray diffraction usually requires approximately six months. The best crystal, with dimensions of 0.2 × 0.02 × 0.04 mm (Fig. 3), was used for data collection.

2.4. Data collection and X-ray diffraction analysis

The crystal was sealed in a glass capillary at room temperature before the diffraction experiment. Data were collected on beamline BL-18B at the Photon Factory in KEK, Tsukaba, Japan using a CCD detector (ADSC Quantum four-dimensional). Synchrotron radiation with a wavelength of 1.0 Å was used in the experiment. A total of 120 images were recorded from 60 to 180° in oscillation angle steps of 1.0°, the exposure time being set to 1 min per frame. The diffraction limit extended beyond 2.0 Å. Intensity data were processed using the *DPS*/*MOSFLM* software (Rossmann & van Beek, 1999). Diffraction analysis showed that the crystal belonged to space group $P2_12_12$, with unit-cell parameters a = 61.087, b = 91.488, c = 81.132 Å. A total of 195 350 reflections to 2.0 Å resolution were merged into 31 377 unique reflections, with an overall $R_{\rm merge}$ of 0.097 and a completeness of 99.8%. $R_{\rm merge}$, $I/\sigma(I)$ and the completeness of the highest resolution shell (2.11–2.0 Å) are 0.296, 2.5 and 99.8%, respectively. The number of monomers in an asymmetric unit may possibly be three, four or five, corresponding to $V_{\rm M}$ values of 3.1, 2.3 and 1.8 Å^3 Da^{-1}, respectively, all of which fall into the reasonable range for globular proteins (Matthews, 1968). The preliminary analysis *via* molecular replacement identified four monomers in an asymmetric unit, with a corresponding solvent content of 46.2%.

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