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Crystallization and preliminary X-ray crystallographic studies on the fungal immunomodulatory protein Fve from the golden needle mushroom (*Flammulina velutipes*)

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The fungal immunomodulatory protein from the edible golden needle mushroom (*Flammulina velutipes*), designated Fve, is a single polypeptide consisting of 114 amino-acid residues. It is believed to trigger the mitogenic proliferation of T lymphocytes and Th1 cytokine production. Here, it is demonstrated that Fve forms a homodimer in nature. In order to understand the relationship between its structure and function, Fve was crystallized using the hanging-drop method; the protein formed well diffracting crystals within 3–5 d in 2.5% PEG 400, 2.0 *M* ammonium sulfate and 0.1 *M* Tris base buffer pH 8.5. The space group of the Fve crystals is either $P4_32_12$ or $P4_12_12$, with unit-cell parameters $a = b = 96.92$, $c = 61.42$ Å. The crystal contains two molecules per asymmetric unit and diffracts to 1.4 Å resolution when exposed to synchrotron radiation.

1. Introduction

Many components from mushrooms have been shown to be potential immunoregulatory agents that induce modulatory effects in immune function such as enhanced resistance against infectious agents (Piraino & Brandt, 1999), increased rejection of malignant cells (Fujimiya *et al.*, 1999) and suppressed immune response of systemic anaphylaxis reactions (Ko *et al.*, 1995). However, the field of pharmacological immunostimulants is still at a very early stage of development compared with that of immunosuppressive drugs. Common immunostimulants that are being used in various clinical applications are Bacille Calmette-Guerin (Kamat & Lamm, 2001), β-glucans such as lentinan (Yoshino *et al.*, 2000), Sizofiran (Shimizu *et al.*, 1992) and polysaccharide-K (Iguchi *et al.*, 2001), glycoprotein from *Klebsiella pneumoniae* (Viland & Blomgren, 1987), bacteria extract from *Streptococcus pyogenes* (Okamoto *et al.*, 1993) and immunostimulating factors from black soybean (Liao *et al.*, 2001) and thymus (Federico *et al.*, 1995). Recently, a new family of fungal immunomodulatory proteins, LZ-8, Fip-*vvo* (Vvo), Fip-*gts* (Gts) and Fip-*fve* (Fve), with similar molecular mass, amino-acid composition and biological activities, were identified and isolated from *Ganoderma lucidium* (Kino *et al.*, 1989), *Volvariella volvacea* (Hsu *et al.*, 1997), *G. tsugae* (Lin *et al.*, 1997) and *Flammulina velutipes* (Ko *et al.*, 1995), respectively.

Fip-*fve* protein, designated Fve for simplicity, is a protein with a molecular weight of 12.7 kDa and an isoelectric point of 6.14 that has been isolated from the fruit bodies of the edible mushroom *F. velutipes*. It is a single polypeptide consisting of 114 amino-acid residues. Analysis of the primary structure of Fve revealed there to be no disulfide bonds. Protein secondary-structure prediction showed one α-helix to be located at the N-terminus, which is then followed by six β-strands. Two loop-like structures are located at the N- and C-termini, respectively (Rost, 2001). The percentages of helix, sheet and loop are 11.3, 42.6 and 46.1%, respectively. Sequence alignment showed Fve to have 63% identity with LZ-8 from *G. lucidium* and 53% with Vvo from *V. volvacea*. Fve is an immunomodulating agent that can stimulate the proliferation of mouse splenocytes and human peripheral blood lymphocytes. It is also capable of enhancing IL-2, IFN-g and TNF-α gene expression. Animal studies have demonstrated that Fve protein prevents systemic anaphylactic reactions and significantly decreases footpad oedema during the Arthus reaction (Ko *et al.*, 1995). Unlike most of the polysaccharides and other immunoregulatory components identified to date, Fve is a small protein, which makes it an ideal candidate for drug design by protein engineering. Therefore, the three-dimensional structure of this fungal immunomodulatory protein would provide a good basis for understanding the protein's functions, immune reactions and therapeutic applications. In this study, we have obtained crystals of the fungal immunomodulatory protein Fve using high-salt crystallization conditions; the crystals diffract to 1.4 Å resolution when exposed to synchrotron radiation.

2. Experiments and results

2.1. Purification of native Fve protein

2 kg of *F. velutipes* fruit bodies were homogenized with 2 l of ice-cold 5% acetic acid in the presence of 50 m*M* 2-mercaptoethanol and 0.3 *M* sodium chloride. The proteins in the supernatant were precipitated with 95% saturated ammonium sulfate. In order to remove ammonium sulfate completely before protein purification by ion-exchange chromatography, the precipitate was redissolved and dialyzed against 10 m*M* Tris–HCl pH 8.5 (buffer *A*) at 277 K for 48 h with six to eight changes of dialysis buffer. Protein solution was applied to a Q Sepharose Fast Flow column (2.6 × 10 cm; Pharmacia) previously equilibrated with buffer *A*. The unbound fraction was collected and dialyzed against 10 m*M* sodium acetate pH 5.0 (buffer *B*) at 277 K for 48 h with six to eight changes of dialysis buffer and then further purified by applying to an SP Sepharose Fast Flow column (2.6 × 10 cm; Pharmacia) previously equilibrated with buffer *B*. The protein was eluted with a gradient of 0–0.5 *M* NaCl in buffer *B*. Fractions containing Fve protein were collected and analyzed by 7.5% Tris–tricine SDS–PAGE. The native Fve protein has an apparent molecular weight of 12.7 kDa as determined by SDS–PAGE (Fig. 1*a*). However, it appeared to be a homodimer with a molecular weight of 25.5 kDa as determined by Superdex 75 (26 × 60 cm; Pharmacia) gel-filtration chromatography (Figs. 1*b* and 1*c*). The running buffer for gel filtration was 10 m*M* Tris–HCl pH 7.5, 0.2 *M* sodium chloride. Purified Fve was further analyzed by MALDI–TOF mass spectrometry. The fingerprints of four tryptic peptides and two peptides with one missing cleavage, representing 54.3% sequence coverage of Fve, were obtained (data not shown).

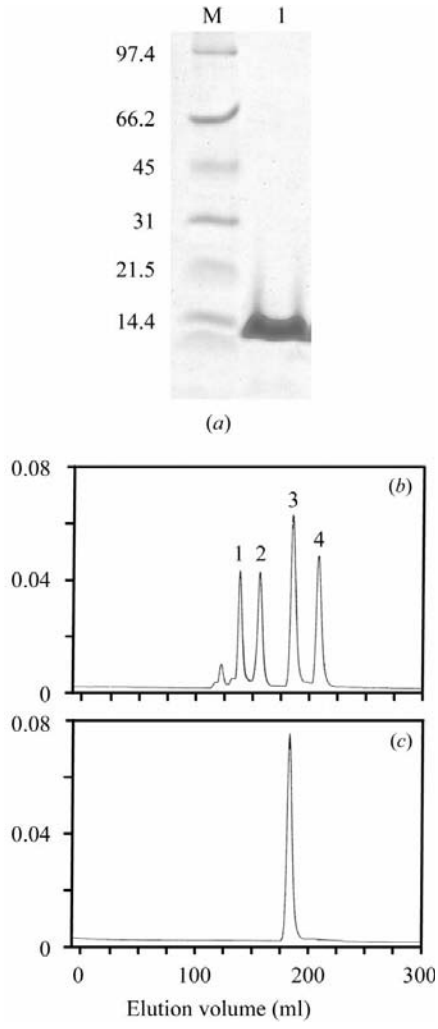

Figure 1
Analysis of purified Fve by SDS–PAGE and gel-filtration chromatography. (*a*) The native Fve protein purified by cation- and anion-exchange chromatography was analyzed by tricine SDS–PAGE. Fve protein gave a single band with an apparent molecular mass of 12.7 kDa. Lane M, molecular-weight markers; lane 1, purified native Fve protein. (*b*) Elution profile of calibration proteins by Superdex 75 chromatography. Peaks: 1, bovine serum albumin (67 kDa); 2, ovalbumin (43 kDa); 3, chymotrypsinogen A (25 kDa); 4, ribonuclease A (13.7 kDa). (*c*) Purified native Fve formed a homodimer of 25.5 kDa.

Fve protein is the major component of the crude extract of the mushroom fruit bodies. By removing the cap of the mushroom, we managed to reduce the amount of polysaccharides that caused undesirable interference in the protein-purification process. The yield of native Fve protein was 40 mg from 1 kg wet weight of starting material.

2.2. Crystallization

Fve protein was concentrated to 4 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.5. Initial crystallization screening was performed using sparse-matrix crystallization screening kits Crystal Screen 1 and 2 from Hampton Research (Jancarik & Kim, 1991; Cudney *et al.*, 1994). All screening and crystal trials used the hanging-drop vapour-diffusion method at 294 K in VDX multi-well plates with 650 µl reservoir solution. Drops consisting of 4 µl reservoir solution and 4 µl protein sample (4 mg ml^{-1}) were equilibrated over the well solution for one week. After extensive screening, plate-like crystals were obtained under two different low-salt conditions: (i) 30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* $MgCl_2$ and (ii) 30% PEG 4000, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* NaOAc. Three-dimensional cubic shaped and octahedral crystals also appeared after 3 d under two different high-salt conditions: (i) 2.0 *M* $(NH_4)_2SO_4$, 0.1 *M* Tris–HCl pH 8.5 and (ii) 2% PEG 400, 0.1 *M* Na HEPES pH 7.5, 2.0 *M* $(NH_4)_2SO_4$. To optimize the crystallization conditions, combinations of varying protein and salt concentrations, different molecular-weight PEGs and different pH values were screened. The high-salt lead conditions were optimized to 2.5% PEG 400, 2.0 *M* $(NH_4)_2SO_4$, 0.1 *M* Tris–HCl pH 8.5 at 294 K. Crystals grew to approximate dimensions of 1.0 × 0.9 × 0.5 mm within 5 d (Fig. 2). All crystals, including large crystals, were frozen. The crystals were cryopreserved by dipping them in mother liquor containing 25% of glycerol for 1 min, after which they were flash-frozen in liquid nitrogen at 100 K and stored in a dewar.

2.3. X-ray analysis

The X-ray diffraction intensities from Fve crystals were measured at 100 K on beam-

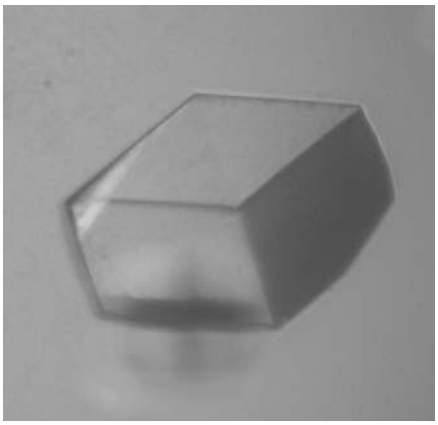

Figure 2
A representative crystal of Fve. The tetragonal crystal was grown in 2% PEG 400, 2.0 *M* ammonium sulfate, 0.1 *M* Tris base pH 8.5. The crystal dimensions are approximately 1 × 0.9 × 0.5 mm.

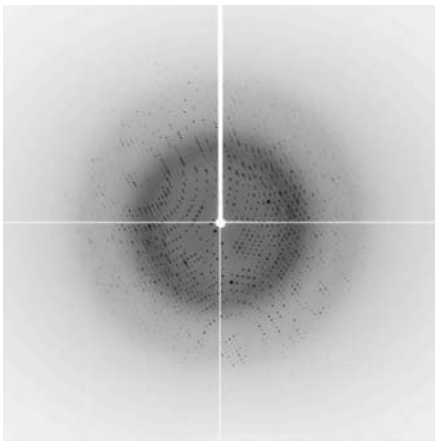

Figure 3
1° oscillation image of Fve crystal. The edge of the image corresponds to a resolution of 1.4 Å. Image displayed with *MOSFLM*/*SCALA*.

Table 1
Data collection and statistics.

Values in parentheses are for the highest resolution shell, 1.49–1.4 Å.

X-ray source, beamline	SSRL, BL9-2
Wavelength (Å)	1.07
Crystal-to-detector distance (mm)	99.97
Unit-cell parameters (Å, °)	a = 96.92, b = 96.92, c = 61.42, $\alpha = \beta = \gamma$ = 90.00
Space group	$P4_32_12$ or $P4_12_12$
No. of molecules per AU	2 (one dimer)
No. of observed reflections	344079
No. unique reflections	56993
Solvent content (%)	56.4
V_M (Å^3 Da^{-1})	2.8
Resolution range (Å)	33.5–1.4 (1.49–1.4)
Average $I/\sigma(I)$	10.1 (1.29)
R_{merge}†	0.047 (0.54)
Completeness (%)	99.7 (93.5)

† $R_{merge} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the mean intensity of symmetry-related measurements of the reflection.

line BL9-2 of the Stanford Synchrotron Radiation Laboratory facility with an ADSC Quantum-315 CCD detector. Data were collected at a wavelength of 1.07 Å. All data were processed using *MOSFLM* (Leslie, 1992) and X-ray intensities were scaled with *SCALA* (Collaborative Computational Project, Number 4, 1994). Well ordered diffraction data at 1.4 Å resolution were collected from larger crystals (Fig. 3). Analysis of the collected data (Table 1) indicated that Fve crystals belong to a tetragonal space group, either $P4_32_12$ or $P4_12_12$, with unit-cell parameters $a = b = 96.92$, $c = 61.42$ Å. The Matthews parameter (V_M) of these crystals is 2.8 Å^3 Da^{-1} and thus the solvent content is 56.4% assuming two molecules (one dimer) of Fve per asymmetric unit (Matthews, 1968). A total of 344 079 observations were obtained at 1.4 Å resolution, giving approximately 56 993 unique reflections (99.7% completeness, R_{merge} = 0.047).

In summary, we have obtained well diffracting crystals of the native fungal immunomodulatory protein Fve from the golden needle mushroom. A search for suitable heavy-atom derivatives is in progress. We are attempting to derivatize Fve with high-pressure xenon gas and NaBr for structure determination. The first three-dimensional structure of Fve is expected to facilitate further studies of the mechanisms of protein–lymphocyte interaction and immunomodulation.

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