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Purification, characterization and preliminary crystallographic studies of a PR-10 protein from *Pachyrrhizus erosus* seeds

Fang Wu,[a,b] Yikun Li,[a,b] Shaojie Chang,[a,b] Zhaocai Zhou,[a,b] Fang Wang,[a,b] Xiaomin Song,[a,b] Yujuan Lin[c] and Weimin Gong[a,b]*

[a]School of Life Sciences, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China, [b]Key Laboratory of Structural Biology, Chinese Academy of Sciences, Hefei, Anhui 230026, People's Republic of China, and [c]Fujian Institute of Matters Structure, Fuzhou, Fujian, People's Republic of China

Correspondence e-mail: wgong@ustc.edu.cn

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A 16 kDa protein SPE16 was purified from the seeds of *Pachyrrhizus erosus*. Its N-terminal amino-acid sequence showed significant sequence homology to pathogenesis-related proteins from the PR-10 family. An activity assay indicated that SPE16 possesses ribonuclease activity as do some other PR-10 proteins. SPE16 crystals were obtained by the hanging-drop vapour-diffusion method. The space group is $P2_12_12_1$, with unit-cell parameters $a = 53.36$, $b = 63.70$, $c = 72.96$ Å.

1. Introduction

Pathogenesis-related (PR) proteins are plant proteins, most of which are induced by biotic (pathogen infection) or abiotic (wounding, dark growth or environmental) stress. The first PR protein was identified in tobacco leaves responding to tobacco mosaic virus (TMV) infection (Gianinazzi *et al.*, 1970; Van Loon & Kammen, 1970). Many similar proteins have now been identified in tobacco as well as in dicots and monocots. These PR proteins were initially divided into five groups according to their serological properties, sequence, cellular localization and isoelectric point (Stintzi *et al.*, 1993).

As more and more PR proteins were identified in various species, Van Loon *et al.* (1994) classified them into 11 families based on their amino-acid sequence, serological relationship and/or enzymatic or biological activities. The PR-10 family has been reported to be ubiquitous in the plant kingdom (Fristensky *et al.*, 1988; Walter *et al.*, 1996; Crowell *et al.*, 1992; Breda *et al.*, 1996; Breiteneder *et al.*, 1993; Vanek-Krebitz *et al.*, 1995). Some PR-10 proteins act as allergens, such as the major birch-pollen allergen Bet v 1 (Breiteneder *et al.*, 1989) and food allergens from apple (Vanek-Krebitz *et al.*, 1995) and celery (Breiteneder *et al.*, 1993).

The PR-10 family proteins are cytosolic, have slightly acidic pIs and a molecular mass of around 17 kDa. They are resistant to proteases (Awade *et al.*, 1991; Warner *et al.*, 1994). Not only can PR-10 proteins be induced by pathogen invasion, wounding, dark or other elicitors, but some of them are also observed to be constitutively expressed in roots, stems (Crowell *et al.*, 1992; Sikorski *et al.*, 1999) and dry seeds (Barratt & Clark, 1993). This implies that PR-10 proteins are not only involved in general defence mechanisms, but that they also play important roles in plant development.

The biochemical functions of PR-10 proteins in plant growth and development are as yet unknown. However, the high amino-acid sequence identity to two ginseng ribonuclease proteins suggested that PR-10 proteins possess ribonuclease activity (Moiseyev *et al.*, 1994). Thus, Van Loon *et al.* (1994) classified PR-10 proteins as ribonuclease-like PR proteins. Furthermore, PR-10 members from Bet v 1 (Bufe *et al.*, 1996) and white lupin (Bantignies *et al.*, 2000) have been reported to show ribonuclease activity. From this family, only the crystal structure of the birch-pollen allergen Bet v 1 has been solved (Gajhede *et al.*, 1996).

Here, we report for the first time the purification, characterization and preliminary crystallographic studies of SPE16, an abundant 16 kDa protein from *Pachyrrhizus erosus* seeds. Sequence analysis indicates that it belongs to the PR-10 family.

2. Materials and methods

2.1. Protein purification

100 g of *P. erosus* seeds (purchased from a local market) were homogenized in 0.02 *M* phosphate buffer pH 7.2 containing 0.1 *M* NaCl and left at 277 K overnight. The mixture was centrifuged at 10 000 rev min^{-1} at 277 K for 20 min and the supernatant was treated with saturated ammonium sulfate. The suspension was centrifuged at 12 000 rev min^{-1} at 277 K for 20 min. The protein pellet was redissolved in 20 m*M* Tris–HCl buffer pH 7.2 and loaded onto a DEAE-Sepharose Column (Amersham Pharmacia Biotech) pre-equilibrated with the same buffer. After extensive washing, SPE16 was eluted with a linear gradient of 0–0.5 *M* NaCl. The subsequent

Superdex G75 size-exclusion chromatography was performed with 50 m*M* Tris–HCl pH 7.5 buffer containing 0.15 *M* NaCl.

2.2. IEF–PAGE

Native IEF–PAGE was performed on a gel plate (Li *et al.*, 1994). A mixed solution of 7% acrylamide containing 2.5% ampholine pH 3.5–10, 0.05% ammonium persulfate and 0.01% TEMED was used to prepare a gel plate. The electrode solutions were 1 *M* NaOH (cathode) and 1 *M* H_3PO_4 (anode).

2.3. N-terminal sequencing

The purified protein was transferred from an SDS–PAGE gel to a PVDF membrane. The N-terminal peptide was sequenced at Hunan Normal University, People's Republic of China.

2.4. RNA-degradation activity assay

RNase activity of the purified SPE16 protein was measured at room temperature. The reaction mixtures contained 10 µg total RNA of *P. erosus* and 10 µg SPE16 protein in 10 m*M* Tris–HCl pH 7.5 buffer. After a period of incubation, the proteins were removed from the reaction mixtures by extraction with phenol–chloroform. As a control, the reaction mixture without SPE16 protein was also incubated for 6 h at room temperature. The experimental results were observed on 1.2% agarose gel. For further determination of the RNase activity of this protein, bovine serum albumin (Sino-American Biotec) was used as a protein sample control.

2.5. Crystallization and X-ray diffraction

SPE16 was crystallized using the hanging-drop vapour-diffusion method at room temperature. The initial crystallization conditions (2.0 *M* ammonium sulfate, 0.1 *M* citrate buffer pH 5.6, 0.2 *M* potassium/sodium tartrate) were obtained using Crystal Screen kits I and II from Hampton Research. Crystals of larger size were obtained by adjusting the concentrations of ammonium sulfate and potassium/sodium tartrate. The diffraction data were collected at 298 K using an in-house X-ray source at a wavelength of 1.5418 Å with a MAR Research image-plate system. The exposure time was 20 min for each image covering 1° of oscillation. Data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The self-rotation function was calculated using the programs *ALMN* (Crowther, 1972) from *CCP*4 (Collaborative Computational Project, Number 4, 1994) and *SELF_RF* from *X-PLOR* (Brünger, 1992)

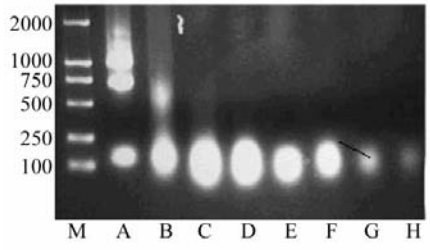

Figure 1
Ribonuclease-activity determination of SPE16. Lane M, molecular-weight markers (bp). Lane A, RNA after 6 h incubation as the control; lane B, RNA (10 µg) + SPE16 (10 µg), 15 s incubation; lanes C–H, RNA + SPE16 with 1–6 h of incubation, respectively.

3. Results

3.1. Protein purification and characterization

SPE16 eluted in the first peak from the DEAE-Sepharose chromatography. The purified protein appeared as a single band with a molecular mass of 16 kDa on SDS–PAGE and was eluted from a Superdex G75 column with an apparent molecular weight of 33 kDa, corresponding to the expected molecular weight of the homodimer. A total of 30 mg of SPE16 was purified from 100 g of dry seeds. Native IEF–PAGE showed that the SPE16 protein is an acidic protein, with a pI of 4.5. The sequence of the first 19 amino acids was GVFVFRDETSSSVAPAKLY. A serach for homologous sequences using *BLAST* 2.0 in the Swiss-Prot database showed the sequence to be similar to the class-10 pathogenesis-related proteins (PR-10). Activity-assay experiments showed that SPE16 possessed ribonuclease activity against total RNA isolated from *P. erosus* (Fig. 1). Bovine serum albumin as a control protein sample did not degrade any of the RNA sample tested after 6 h incubation at room temperature.

3.2. Preliminary crystallographic studies

The best crystals grew with a well solution containing 1.8 *M* ammonium sulfate, 0.1 *M* citrate buffer pH 5.6, 0.05 *M* potassium/sodium tartrate and were good enough for diffraction data collection. The protein concentration was set to 15 mg ml^{-1}. The crystal diffracted to 3.0 Å resolution. Symmetry and systematically absent reflections suggested that the SPE16 crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 53.36, b = 63.70, c = 72.96 Å (Table 1). There could be one or two SPE16 subunits in a crystallographic asymmetric unit, with corresponding V_M values of 3.8 or 1.9 Å^3 Da^{-1}, respectively. However, no obvious self-rotation peaks were found in the Patterson map.

Table 1
Summary of diffraction data collection and processing.

Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 53.36, b = 63.70, c = 72.96
Temperature (K)	298
Resolution (Å)	3.0
Last shell resolution (Å)	3.07–3.0
Total No. of reflections (>0σ)	29062
No. of independent reflections (>0σ)	5345
Completeness (%)	90.6 (93.6)
R_{merge} (last shell)	0.136 (0.323)

4. Discussion

N-terminal amino-acid sequence comparison showed that SPE16 could be a new member of the class-10 pathogenesis-related proteins and the activity assays showed that SPE16 is likely to have RNase activity. However, a previous report demonstrated that Bet v 1 can bind several types of ligands, suggesting a role that is difficult to relate to RNase activity (Mogensen *et al.*, 2002). Current experiments still cannot completely rule out the possibility that the observed RNase activity is attributable to contamination. On the other hand, as SPE16 was purified from healthy dry *P. erosus* seeds, it is expected to be a constitutive protein rather than a protein induced by pathogen invasion or other elicitors as is the case with many previously reported PR-10 proteins. The role of SPE16 in plant developmental regulation needs to be further investigated.

SPE16 is a homodimer in solution, consisting of two 16 kDa monomers. Only the major birch-pollen allergen Bet v 1 has previously been reported as either a monomer or a dimer (Bufe *et al.*, 1996). No other PR-10 family members have been observed as dimers. Whether this dimer formation is functionally significant is under investigation.

Recently, the cDNA coding for SPE16 protein has been cloned in our laboratory (details to be published elsewhere). The amino-acid sequence identity to homologous PR-10 proteins from other plants ranges from 24 to 66%, which further suggests that SPE16 belongs to the PR-10 family.

The three-dimensional structure of the major birch-pollen allergen Bet v 1 has been determined by X-ray diffraction and NMR

spectroscopy (Gajhede *et al.*, 1996). No other PR-10 proteins structures are available at present. The successful crystallization of SPE16 has provided a good system for the study of the RNase mechanisms of PR-10 proteins.

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