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Purification, characterization and preliminary crystallographic studies of a novel plant defensin from *Pachyrrhizus erosus* seeds

Xiaomin Song,[a,b] Zhaocai Zhou,[a,b] Jing Wang,[a,b] Fang Wu[a,b] and Weimin Gong[a]*

[a]National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, People's Republic of China, and [b]School of Life Sciences, University of Science and Technology of China, Key Laboratory of Structural Biology, Chinese Academy of Sciences, People's Republic of China

Correspondence e-mail: wgong@sun5.ibp.ac.cn

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Defensins are small cysteine-rich antimicrobial peptides that are widely distributed in plants, insects and mammals. As potent defenders in protecting plants from pathogenic fungal attack, plant defensins are presumed to play an important role in the innate immunity of plants and are expected to find applications in the production of transgenic crops. A novel plant defensin protein SPE10 from *Pachyrrhizus erosus* seeds was purified and partially sequenced. Crystallization screening using the hanging-drop vapour-diffusion method resulted in two crystal forms, from one of which a diffraction data set was collected to 0.98 Å resolution. The crystal belongs to space group $P2_1$, with unit-cell parameters a = 32.71, b = 28.11, c = 54.85 Å, β = 103.78°. Preliminary crystallographic studies revealed two subunits in the asymmetric unit.

1. Introduction

Plant defensins are characterized as highly basic and cysteine-rich small (4–6 kDa) peptides that are capable of inhibiting the growth of a broad range of fungi and/or bacteria. At the beginning of the 1990s, plant defensins were first isolated from wheat and barley grains (Colilla *et al.*, 1990; Mendez *et al.*, 1990). Following their initial identification in seeds, plant defensins have also been identified in flower organs, leaves, pods, fruit and tubers. It has been demonstrated that many plant defensins are inducible by pathogen challenge (Penninckx *et al.*, 1996). This inducible character, together with the fact that they are mainly expressed in the peripheral cell layer, suggests that plant defensins are involved in the first line of defence against pathogen attack. In fact, defensins are considered to be the only class of peptides that are conserved among plants, invertebrates and vertebrates in the innate immune response (Thomma *et al.*, 2002).

In addition to antifungal activity, some plant defensins exert different functions such as antibacterial activities (Moreno *et al.*, 1994; Osborn *et al.*, 1995; Segura *et al.*, 1998), and proteinase (Wijaya *et al.*, 2000) and insect gut α-amylase (Bloch & Richardson, 1991) inhibitory activities. As their antifungal activities have been extensively studied, many efforts have been made to elucidate the antifungal mechanism and several hypotheses have been proposed (Thomma *et al.*, 2002). Although mammalian and insect defensins are believed to work by directly interacting with plasma-membrane phospholipids, plant defensins seem to interact with a specific membrane receptor, which changes the membrane structure and permeability (Thevissen *et al.*, 1996, 1999) or induces distinct signalling pathways (Thevissen *et al.*, 1997). However, little is known about the putative receptors and the possible molecular components involved in the signalling pathway (if there is one) for most plant defensins.

Unlike phytoalexin, plant defensins seem to be non-toxic to mammalian and plant cells, which qualifies them as good candidates for transgenic application. In fact, some of them have been utilized successfully in producing transgenic crops with improved pathogen resistance (Punja, 2001; Osusky *et al.*, 2000). On the other hand, defensins provide an ideal model for the design of new peptide drugs owing to their small, rigid and compact construction, as well as their varying functions. These important roles warrant further studies on the three-dimensional structures of defensins in order to obtain deeper insights into their structure–function relationships.

To our knowledge, no crystal structure of a plant defensin is presently available, although several structures have been determined by the NMR method (Almeida *et al.*, 2002; Bloch *et al.*, 1998; Bruix *et al.*, 1995; Fant *et al.*, 1998, 1999; Fung *et al.*, 2003). Here, we report for the first time the purification, characterization and preliminary crystallographic studies of SPE10, a novel constitutively expressed defensin from *Pachyrrhizus erosus* seeds.

2. Experimental methods

100 g of *P. erosus* seeds were homogenized in 0.02 *M* phosphate buffer pH 7.2 containing 0.1 *M* NaCl and then extracted overnight. The

extract was filtered and the pH of the filtrate was adjusted to 4.0 with 50%(*v*/*v*) acetic acid. After being stirred slowly for 4 h, the mixture was centrifuged at 10 000 rev min^{-1} for 40 min. In order to reduce the accompanying precipitate, which greatly impairs subsequent crystal growth, the protein components were precipitated with saturated ammonium sulfate and the pellet was redissolved in 20 m*M* Tris–HCl pH 7.5 buffer. After desalting by ultrafiltration, the sample was loaded onto a DEAE-Sepharose column (Amersham Pharmacia Biotech) pre-equilibrated with the same buffer. Electrophoresis showed that SPE10 was eluted in the flowthrough peak. The corresponding fractions were pooled, concentrated and applied onto a Sephacryl-100 column pre-equilibrated with a buffer consisting of 50 m*M* Tris–HCl pH 7.5 and 0.15 *M* NaCl using an ÄKTA FPLC system (Amersham-Pharmacia). Fractions containing SPE10 were collected and prepared for subsequent study with a final concentration of 32 mg ml^{-1} (determined by the Bio-Rad protein assay with BSA as the standard marker). All purification steps were performed at 277 K.

The purified protein was applied onto a reverse-phase C-18 HPLC column (Pharmacia) using eluents buffer *A* (0.1% trifluoroacetic acid) and buffer *B* (acetonitrile containing 0.1% trifluoroacetic acid). The column was eluted with a flow rate of 1 ml min^{-1} and the absorbance at 214 nm was monitored. Only one peak was observed and was collected manually. The sample was then vacuum-dried for sequencing and mass-spectroscopy experiments. N-terminal sequencing was performed at Peking University. The protein-sequence homology was analyzed using a BLAST database search (http://www.ncbi.nlm.nih.gov/BLAST/). Mass-spectrometric analysis was carried out on a Bruker Biflex III mass spectrometer equipped with a 337 nm nitrogen laser.

3. Crystallization and X-ray diffraction

SPE10 was crystallized using the hanging-drop vapour-diffusion method at room temperature. An initial screen using Crystal Screens I and II from Hampton Research gave rise to needle-like crystals using 10%(*v*/*v*) PEG 8000, 0.1 *M* MES pH 6.5 and 0.2 *M* zinc acetate. After optimization, thin plate-shaped crystals were obtained, but showed rather poor diffraction (Fig. 1*a*). A cheap, fast and effective initial screen developed by Michael B. Berry (unpublished work) was also tried and produced better crystals using 35%(*v*/*v*) PEG 8000 with 0.1 *M* HEPES pH 7.0. By further adjusting the pH value and PEG 8000 concentration, larger crystals were obtained that diffracted well (Fig. 1*b*).

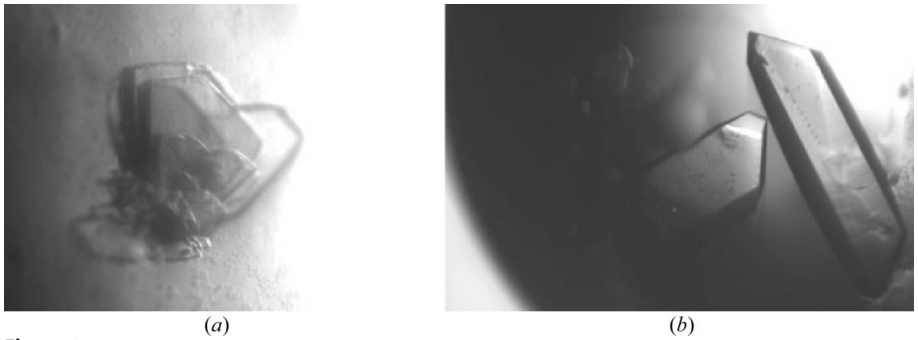
A diffraction data set with excellent quality was collected to 0.98 Å resolution at 100 K using the synchrotron-radiation light source (wavelength 0.9 Å) at the Institute of High Energy Physics, Chinese Academy of Sciences equipped with a MAR CCD area detector. No additional cryoprotectant was used because of the high precipitant concentration. To avoid overexposure of the low reflecting-angle area, two data sets were collected with a 2θ angle of 10°. The first data set was designed specially for high-resolution reflections, with an exposure time of 60 s per image. After 360 images, the exposure time was changed to 0.8 s and a low-resolution data set was then collected.

4. Results

4.1. Protein purification and characterization

After Sephacryl 100 chromatography, SPE10 appeared as a single band with a molecular weight of 10 kDa on SDS–PAGE and of 5 kDa when DTT was added to the loading buffer (Fig. 2). This 10 kDa band may be a dimeric form of SPE10. An alternative explanation is that a small unreduced protein containing disulfide bridges (like SPE10) may not bind optimal amounts of SDS, leading to an inaccurate estimation of the molecular weight (Broekaert *et al.*, 1992). Mass-spectrometric analysis was also carried out to measure the accurate molecular weight of SPE10. The MS results suggest that SPE10 has a molecular weight of 5.49 kDa, suggesting that SPE10 contains about 50 amino acids.

N-terminal sequencing was performed with the result KTCENLADTFRGPCFTDGSCDDHCKNKE. A search for an homologous sequence in the SWISS-PROT database showed that SPE10 has considerable homology with plant defensin proteins. Alignment of SPE10 with these defensins revealed the presence of several conserved residues that are supposed to be essential for this family. As illustrated in Fig. 3, these conserved residues in the mature protein include four cysteine resides at positions 3, 14, 20 and 24, a glycine at position 12, an aromatic residue at position 10, a hydrophobic residue at position 13 and a glutamic acid at position 28 (numbering relative to SPE10).



(*a*) (*b*)

Figure 1
Two crystal forms of SPE10. (*a*) A typical crystal obtained from 10%(*v*/*v*) PEG 8000, 0.1 *M* MES pH 6.5, 0.2 *M* zinc acetate. (*b*) Crystals grown in 35%(*v*/*v*) PEG 8000, 0.1 *M* HEPES pH 7.0. The diffraction data were finally collected using crystal form *B*.

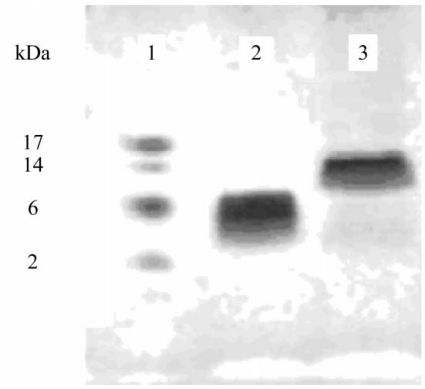


Figure 2
SDS–PAGE analysis of the purified SPE10. Lane 1, molecular-weight markers; lane 2, reduced SPE10; lane 3, non-reduced SPE10.

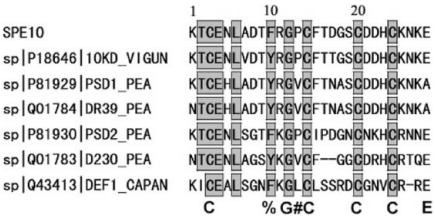


Figure 3
Multiple alignment of the N-terminal amino-acid sequence of SPE10 with homologous plant defensins. Conserved residues are shown in black boxes, while the plant defensin consensus sequence is shown below the alignment. This alignment was performed with *CLUSTALX* (Thompson *et al.*, 1994). The following symbols are used to classify conserved amino acids: %, aromatic residues; #, hydrophobic residues.

4.2. Crystallization and preliminary crystallographic studies

Two crystal forms, form *A* and form *B* (Fig. 1), were obtained, but only form *B* could be used for 0.98 Å data collection. Interestingly, it seems that high concentrations of both protein (up to ∼30 mg ml^{-1}) and precipitant (up to 35% PEG 8000) are preferred for the production of high-quality crystals of SPE10. Additionally, in trials to obtain heavy-atom derivatives, increasing the concentration of PEG 8000 to as high as 40–45%(*v*/*v*) effectively protects the crystals from damage by the infusion of heavy atoms.

For such a small protein as SPE10, reflections in the low-resolution shell are inherently few in number. Thus, overexposed reflections present in the low-resolution shell will significantly impair the overall quality of the diffraction data. To eliminate the effect of overexposure, two data sets were collected at high and low resolution with resolution limits 2.2–0.98 Å and 100–1.5 Å, respectively. Diffraction data were processed with *DENZO* and combined together using the *SCALEPACK* package (Otwinowski & Minor, 1997). Systematic absence analysis suggested that the SPE10 crystals belong to space group $P2_1$, with unit-cell parameters a = 32.71, b = 28.11, c = 54.85 Å, β = 103.78°. Details of the data-collection and processing statistics are given in Table 1. There are two monomers in the crystallographic asymmetric unit, with a V_M value of 2.193 Å^3 Da^{-1}. The self-rotation function was calculated using the *CNS* package (Brünger *et al.*, 1998), with the result ψ = 86.6, φ = −95.9, κ = 36.0°, suggesting an unusual non-crystallographic symmetry between the two subunits.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last shell.

Space group	$P2_1$		
Unit-cell parameters (Å, °)	a = 32.71, b = 28.11, c = 54.85, β = 103.78		
Temperature (K)	100		
Wavelength (Å)	0.90		
Oscillation step (°)	1		
Crystal-to-detector distance (mm)	50		
Resolution limits (Å)	2.2–0.98	100–1.50	100–0.98
Exposure time per image (s)	60	0.8	—
Total reflections	906821	103609	66264
Independent reflections	50541	15739	55557
Completeness (%)	99.5 (96.6)	99.5 (99.9)	99.3 (96.3)
Completeness [$I/\sigma(I) > 3$] (%)	84.15 (47.02)	90.11 (77.45)	85.40 (50.56)
$I/\sigma(I)$	30.54 (4.07)	26.29 (9.39)	31.83 (4.48)
R_{merge}†	0.069 (0.486)	0.043 (0.132)	0.028 (0.000)

† $R_{merge} = \sum_{hkl}\sum_j |I(hkl)_j - \langle I(hkl)\rangle| / \sum_{hkl}\sum_j I(hkl)_j$, where $I(hkl)_j$ is the jth measurement of the intensity of reflection hkl and $\langle I(hkl)\rangle$ is the mean intensity of reflection hkl.

5. Discussion

A new plant defensin protein, SPE10 from *P. erosus* seeds, has been characterized, purified and crystallized. It has been demonstrated that some plant defensins can be expressed constitutively and may contribute to the host's pre-existing constitutive defence arsenal (Wang *et al.*, 1999). This is supported by the fact that SPE10 is expressed at high levels under unchallenged conditions.

SPE10 shares 81% identity with Psd1. It is believed that Psd1 acts as a potassium-channel inhibitor, based on the similarity of its surface-charge distribution to that of several neurotoxins (Almeida *et al.*, 2002). Residues Trp38, Phe15, Lys27 and Val13 of Psd1 are proposed to form a putative interaction site for membrane receptors. Equivalent residues are also present in SPE10 (Lys27 and Phe15), indicating a resemblance in function between SPE10 and Psd1. However, Val13 of Psd1 is replaced with Pro13 in SPE10 and six additional residues are also substituted in the sequenced N-terminal 28 amino acids of SPE10. These substitutions may lead to significant differences in their activities, as is the case in Rs-AFP1 and Rs-AFP2. Differences at only two positions in their primary sequences make Rs-AFP2 2–30 times more active than Rs-AFP1 (Terras *et al.*, 1992).

The mechanism of action of plant defensins has been under continual study since their initial identification. Recently, research has incresingly supported the perspective that plant defensins act *via* specific interaction with a membrane receptor. Mutation analysis in radish defensin identified two possible sites for interaction with a potential receptor (De Samblanx *et al.*, 1997) and a putative binding target, glucosylceramide, has recently been identified as a membrane receptor for Rs-AFP2 (Thevissen *et al.*, 2004), supporting a two-step hypothesis in which defensins first bind to a specific membrane receptor and then permeabilize the membrane in a still unclear mode. Despite these findings, the accurate mode of interaction between defensin and its receptor still remains to be established.

The determination of the SPE10 crystal structure at ultrahigh resolution, as well as systematic comparisons of all related structures (to date, only NMR structures are available), may provide novel insights into the mode of action of plant defensin towards the cell membrane.

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