Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary crystallographic studies of an antimicrobial protein from *Pharbitis nil*

Sung Chul Ha,[a] Kyeongsik Min,[a] Ja Choon Koo,[a] Yeongkyoo Kim,[b] Dae Jin Yun,[a] Moo Je Cho[a] and Kyeong Kyu Kim[a,c]*

[a]Plant Molecular Biology and Biotechnology Research Center, Gyeongsang National University, Chinju 660-701, South Korea, [b]Department of Geology, Kyungpook National University, Daegu 702-701, South Korea, and [c]Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea

Correspondence e-mail: kkim@med.skku.ac.kr

Received 11 August 2000
Accepted 23 October 2000

An antimicrobial protein from seeds of *Pharbitis nil* (Pn-AMP) which shows an antifungal activity towards several agriculturally important plant pathogens has been crystallized in the presence of equimolar *N*-acetylglucosamine with sodium citrate as precipitant. The crystal belongs to the hexagonal space group $P6_122$ (or $P6_522$), with unit-cell parameters $a = b = 29.33$ (5), $c = 133.44$ (12) Å. Native data were collected using a crystal at 100 K to a resolution of 1.78 Å.

1. Introduction

In order to protect themselves from pathogenic invasion, plants produce many antimicrobial proteins such as chitinases, β-1,3-glucanases, ribosome-inactivating proteins and cysteine/glycine-rich small antimicrobial proteins (Stintzi *et al.*, 1993). One of the small antimicrobial proteins, hevein, is a small cysteine-rich chitin-binding protein from the rubber tree (*Hevea brasiliensis*). The 43-residue sequence of hevein, known as the hevein domain, contains eight cysteine residues that are all involved in disulfide bridges with a unique topological arrangement ($CX_8CX_4CCX_5CX_6CX_3CX_3C$; Fig. 1). This domain has so far been found in a wide array of unrelated fungal and plant proteins. Aside from hevein and hevein-like proteins which consist of single copy of the domain itself, the chitin-binding proteins such as wheat-germ agglutinin and plant endochitinases are composed of multiple repeated hevein domains. Interestingly, yeast killer toxin and *Kluyveromyces lactis* killer toxin also contain a hevein domain in the central section of the protein (Fig. 1). Although the hevein domain is found in many plant proteins, it is not known how hevein and hevein-like proteins exhibit antifungal and/or antibacterial activities at the molecular level. It has been suggested that the mode of action of chitin-binding lectins involves the proteins penetrating into the cell and affecting the cell-wall synthesis of fungi that contain chitin. Cell penetration by the hevein domain has also been proposed from the fact that the hevein domain-containing class I chitinases show significantly stronger *in vitro* antifungal activity than the class II chitinases lacking the domain (Iseli *et al.*, 1993). From these observations, it is believed that the capability for chitin binding may play an important role in the antifungal activities of hevein and hevein-like proteins.

Structural studies of hevein (Rodriguez-Romero *et al.*, 1991; Asensio *et al.*, 1998), of wheat-germ agglutinin (Wright, 1987) and of *Urtica dioica* agglutinin (Harata & Muraki, 2000; Saul *et al.*, 2000) revealed that hevein domains have identical disulfide-bridge pairing as well as similar tertiary structures. Antimicrobial protein from *Amaranthus caudatus*, which contains only three cystines, also has a similar fold to that of hevein (Martins *et al.*,

```
Pn-AMP1       QQCGRQASGRLCGNR..LCCSQWG.YC.GSTASYCGA..........GCQS.QCRS  1/41
Hevein        EQCGRQAGGKLCPNN..LCCSQWG.WC.GSTDEYCSP.........DHNCQS.NCKD  1/43
Ac-AMP2     VGECVR...GR.CPSG..MCCSQFG.YC.GKGPKYCGR                      1/30
Barley CHI    QQCGSQAGGATCPNC..LCCSRFG.YC.GSTSDYCG..........AGCQS.QCSG-  28/70
CBP20         QQCGSQAGGATCPNC..LCCSRFG.YC.GSTPEYCSP.........SQGCQS.QCSG-  23/66
UDA           QRCGSQGGGGTCPAL..WCCSIWG.WC.GDSEPYCGR..........TCEN.KCWS-  1/41
WGA3          QRCGEQGSGMECPNN..LCCSQYG.YC.GMGGDYCGK..........GCQNGACWT-  1/42
Allegen R   LVPCA..WAGNVCGEKRAYCCSDPGRYCPWQVVCYESS..........EICSK.KCGK  1/45
Sn-HLPf   -GPWQCGRDAGGALCHDN..LCCSFWG.FC.GSTYQYCED..........GCQS.QCRD-  27/69
Killer Toxin  -PLAPG.EKYNAKCPLN..ACCSEFG.FC.GLTKDYCDKKSSTTGAPGTDGCFS.NCGY-  321/372
```

Figure 1
Comparison of the amino-acid sequence of Pn-AMP1 with other hevein-like proteins and yeast killer toxin. The sequences aligned are from rubber latex hevein (Hevein), antimicrobial peptide from *A. caudatus* (Ac-AMP2), barley chitinase (Barley CHI), tobacco antifungal protein (CBP20), *U. dioica* agglutinin (UDA), wheatgerm agglutinin 3 isolectin (WGA3), pollen allergen RA5 from *Ambrosia elatior* (Allegen R), antimicrobial peptide from elderberry fruit (Sn-HLPf) and *K. lactis* killer toxin α-subunit (Killer Toxin). The numbers on the right indicate the first and last amino acids used in the alignment.

1996). However, current structural data cannot give any further clues to understanding the antifungal activity of hevein-like proteins. In addition, a high-resolution structure is essential to clarify the protein–chitin interaction which is presumed to be important for antifungal activity.

Two antimicrobial proteins (Pn-AMP1 and Pn-AMP2) were isolated from morning glory seeds (*P. nil*; Koo *et al.*, 1998). The amino-acid sequences of Pn-AMP1 and Pn-AMP2 are identical except that Pn-AMP1 has an additional serine residue at the carboxyl-terminus. Pn-AMP1 has 77% sequence identity to hevein and showed potent antifungal activity against a broad spectrum of fungi, including fungi that do not contain chitin in their cell walls (Koo *et al.*, 1998). Since it is known that Pn-AMPs shows a similar fungicidal effect to thionin by disrupting the fungal membrane and causing the leakage of cytoplasmic materials, they might have a different mode of antimicrobial action to hevein (Koo *et al.*, 1998). To elucidate a molecular mechanism for chitin binding and antifungal properties of Pn-AMP that would differ from that of hevein, high-resolution structural study is necessary. In addition, structural study of Pn-AMP could elucidate the implications of sequence conservation among yeast killer toxin and hevein-like proteins. As a first step in structural study, Pn-AMP1 has been crystallized in the presence of *N*-acetyl glucosamine and its preliminary crystallographic data have been analyzed.

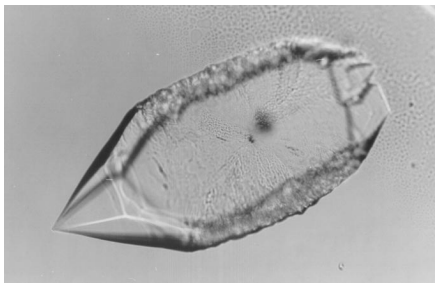

Figure 2
Hexagonal crystal of Pn-AMP1 grown in the presence of equimolar *N*-acetylglucosamine from citrate solution as the precipitant at 277 K. The approximate dimensions of the crystal are 0.2 × 0.2 × 0.5 mm.

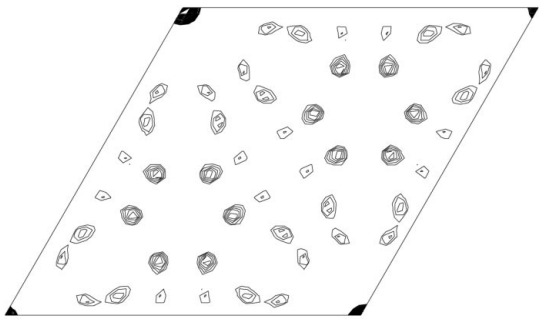

Figure 3
Anomalous Patterson map on Harker section (*UV*0) in hexagonal space group $P6_122$ or $P6_522$. The Patterson map was calculated using data in the 3.0–1.78 Å resolution shell and was contoured at increments of 0.5σ from 3σ.

2. Purification and crystallization

Pn-AMP was purified from morning glory seeds as described by Koo *et al.* (1998) with minor modifications. A crude extract prepared by ammonium sulfate precipitation and heat treatment (Koo *et al.*, 1998) was applied onto a CM-Sepharose (Pharmacia) column equilibrated with 10 m*M* sodium phosphate buffer pH 6.0 and was eluted with 500 m*M* NaCl. Fractions containing antifungal activity were pooled and applied to a reverse-phase column (C18 silica, SEP-PAK, Waters, Milford, MA, USA) and eluted with 50% acetonitrile in 0.1% trifluoroacetic acid (TFA). The eluted protein fractions were lyophilized and dissolved in 400 ml of sterile water and subjected to reverse-phase HPLC on a Zorbax C18 column (4.6 × 150 mm, Hewlett Packard). The protein peak containing Pn-AMP1 was eluted with a linear gradient of 0–70% acetonitrile and dried in a vacuum concentrator. Pn-AMP1 was dissolved in water at a concentration of 25 mg ml^{-1} for crystallization purposes. Initial crystallization conditions were tested using the Hampton Research Crystal Screens I and II using the hanging-drop vapour-diffusion method. 1 µl of protein solution was mixed with an equal volume of reservoir solution and equilibrated against 500 µl of reservoir solution at 295 and 277 K. The same crystallization experiments were performed using the protein solution containing *N*-acetylglucosamine, the monomer unit of chitin, in a 1:1 molar ratio. Hexagonal rod-shaped crystals were obtained with 0.64 *M* sodium citrate pH 6.2 as precipitant and equimolar *N*-acetylglucosamine in the protein drop at 277 K. Crystals grew to dimensions of 0.2 × 0.2 × 0.5 mm within a month (Fig. 2). Since crystals were only obtained in the presence of *N*-acetylglucosamine, it can be assumed that *N*-acetylglucosamine is essential for the crystallization of Pn-AMP.

Table 1
Synchrotron data-collection statistics.

Values in parentheses refer to the highest resolution bin.

Space group	$P6_122$ or $P6_522$
Unit-cell parameters (Å,°)	$a = b = 29.33$ (5), $c = 133.44$ (12), $\alpha = \beta = 90$, $\gamma = 120$
Resolution (Å)	50.0–1.78 (1.84–1.78)
No. of unique reflections	3577 (293)
Redundancy	7.38 (3.10)
$R_{\rm merge}$† (%)	7.8 (16.5)
$R_{\rm ano}$‡ (%)	5.3 (11.8)
Completeness (%)	93.9 (79.2)
$I/\sigma(I)$	32.9 (7.9)
Temperature (K)	100

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$. ‡ $R_{\rm ano} = |F(+) - F(-)|/\langle F\rangle$.

3. Data collection and processing

Cryocooling was required for collection of the complete data set owing to the radiation sensitivity of the crystals. Individual crystals soaked in a reservoir solution containing 30% glycerol were scooped up in a cryo-loop and frozen in a nitrogen stream at 100 K. The first set of X-ray data was collected to 3.0 Å from a frozen crystal of Pn-AMP1 on a MacScience 2030b area detector with mirror-focused Cu $K\alpha$ X-rays ($\lambda = 1.5418$ Å) from a MacScience M18XHF rotating-anode generator operated at 50 kV and 90 mA. The merged data set consists of 769 unique reflections with an $R_{\rm merge}$ of 15.5% and 90% completeness. X-ray diffraction data were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL* program suite (Otwinowski & Minor, 1997). The crystal belongs to the hexagonal space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 29.33$ (5), $c = 133.44$ (12) Å. Assuming a molecular weight of 4300 Da and one molecule in the asymmetric unit, the value of the crystal packing parameter ($V_{\rm M}$) is 1.93 Å^3 Da^{-1} and the solvent content is 39% (Matthews, 1968).

To obtain a higher resolution crystal structure for defining the interaction between Pn-AMP1 and *N*-acetylglucosamine, a second data set was collected using a Weisenberg camera for macromolecular crystallography at the BL-6A experimental station of the Photon Factory, Tsukuba, Japan (Sakabe, 1991). To maximize the anomalous contribution of the S atoms to phasing, the wavelength for the data collection was selected to be 1.800 Å; *i.e.* the lowest energy available at the beamline. The diffraction patterns recorded on image plates (200 × 400 mm, Fuji BASIII) were digitized by the off-line scanner BA100. The oscillation range per image plate was 5.5°, with an overlap of 0.5°. For measuring redundant and reliable

anomalous signals from eight S atoms in Pn-AMP1, a full sphere was covered from a frozen crystal using a crystal-to-detector distance of 286.5 mm and a 0.1 mm collimator. X-ray diffraction data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The synchrotron data were collected from 50.0 to 1.78 Å with an $R_{\rm merge}$ of 7.8% and an $R_{\rm ano}$ of 5.3%. Data statistics are summarized in Table 1.

4. Anomalous Patterson map

Since eight out of 41 residues are cysteine in Pn-AMP1, the anomalous signal from the S atoms could be useful for improving the phase and identifying the four cystines in the model. Single-wavelength anomalous diffraction data were collected at 1.800 Å, where the anomalous scattering coefficient f'' is 0.75 e. When reflection data in the 3.0–1.78 Å resolution shell were used for calculation, four anomalous Patterson peaks that might correspond to the four cystine residues were identified in the Harker section $w = 0$ with peak intensities of 4.84, 3.78, 3.64 and 3.18σ (Fig. 3). Further studies will be undertaken in order to find further Patterson peaks necessary to identify the cystine positions.

The crystal structure determination of the Pn-AMP1 by molecular replacement combined with anomalous dispersion will elucidate the atomic details of chitin binding and the possible antifungal activity of Pn-AMP.

We thank Professor N. Sakabe and Dr M. Suzuki for assistance during data collection at NL-6A of the Photon Factory, Japan. This work was supported by a grant from the Genetic Engineering Program (1998) of Korea Research Foundation.

References

Asensio, J. L., Canada, F. J., Bruix, M., Gonzalez, C., Khiar, N., Rodriguez-Romero, A. & Jimenez-Barbero, J. (1998). *Glycobiology*, **8**, 569–577.

Harata, K. & Muraki, M. (2000). *J. Mol. Biol.* **297**, 673–681.

Iseli, B., Boller, T. & Neuhaus, J. M. (1993). *Plant Physiol.* **101**, 857–863.

Koo, J. C., Lee, S. Y., Chun, H. J., Cheong, Y. H., Choi, J. S., Kawabata, S., Miyagi, M., Tsunasawa, S., Ha, K. S., Bae, D. W., Han, C., Lee, B. L. & Cho, M. J. (1998). *Biochem. Biophys. Acta*, **1382**, 80–90.

Martins, J. C., Maes, D., Loris, R., Pepermans, H. A., Wyns, L., Willem, R. & Verheyden, P. (1996). *J. Mol. Biol.* **258**, 322–333.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Rodriguez-Romero, A., Ravichandran, K. G. & Soriano-Garcia, M. (1991). *FEBS Lett.* **291**, 307–309.

Sakabe, N. (1991). *Nucl. Instrum. Methods A*, **303**, 448–463.

Saul, F. A., Rovira, P., Boulot, G., Damme, E. J., Peumans, W. J., Truffa-Bachi, P. & Bentley, G. A. (2000). *Structure Fold. Des.* **8**, 593–603.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M. & Fritig, B. (1993). *Biochimie*, **75**, 687–706.

Wright, C. S. (1987). *J. Mol. Biol.* **194**, 501–529.