Crystal structure of a novel antifungal protein distinct with five disulfide bridges from *Eucommia ulmoides* Oliver at an atomic resolution

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**Abstract**

EAFP2 is a novel antifungal protein isolated from the bark of the tree *Eucommia ulmoides* Oliver. It consists of 41 residues and is characterized with a five-disulfide motif and the inhibitory effects on the growth of both cell wall chitin-containing and chitin-free fungi. The crystal structure of EAFP2 at an atomic resolution of 0.84 Å has been determined by using *Shake-and-Bake* direct methods with the program *SnB*. The phases obtained were of sufficient quality to permit the initial model built automatically and the structural refinement carried out using anisotropic displacement parameters resulted in a final crystallographic *R* factor of 6.8%. In the resulting structural model, all non-hydrogen protein atoms including an unusual pyroglutamyl acid residue at the N-terminal can fit to the articulated electron densities with one centre and more than 65% of the hydrogen atoms in the protein can be observed as individual peaks in the difference map. The general fold of EAFP2 is composed of a 310 helix (Cys3–Arg6), an *α*-helix (Ala27–Cys31) and a three-stranded antiparallel β-sheet (Cys16–Ser18, Cys23–Ser25, and Cys35–Cys37) and cross-linked by five disulfide bridges. The tertiary structure of EAFP2 can be divided into two structural sectors, A and B. Sector A composed of residues 11–30 adopts a conformation similar to the chitin-binding domain in the hevein-like proteins and features a hydrophobic surface embraced a chitin-binding site (Tyr20, 22, 29, and Ser18). The distinct disulfide bridge Cys7–Cys37 connects the N-terminal ten residues with the C-terminal segment 35–41 to form the sector B, which features a cationic surface distributing all four positively charged residues, Arg6, 9, 36, and 40. Based on these structural features, the possible structural basis of the functional properties of EAFP2 is discussed.

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**Keywords:** *Eucommia* antifungal protein; Atomic resolution crystallography; Direct methods; Five-disulfide-bond motif; Structure–function relationship

1. Introduction

Over the past 20 years a wide array of antimicrobial peptides and proteins have been discovered throughout plant and animal kingdoms, which provide insights into the innate defensive system and attract increasing attention recently (Zasloff, 2002). It is well known that plants have no immune system but can live harmony with various microbial pathogens. They have evolved highly effective defense mechanisms to prevent the invasion of microorganisms. The wide spread antimicrobial peptides or proteins, produced either in a constitutive or in an inducible manner, are believed to be involved in these mechanisms (Broekaert et al., 1997; Fritig et al., 1998). So far a lot of plant peptides with antimicrobial activities in vitro have been identified. They are highly divergent in their sequences and show quite different antimicrobial activities. To date the mode of action for these peptides is still much debated and, therefore, it is essential to provide the three-dimensional structures of the respective antimicrobial peptides for uncovering the molecular mechanism. Among the vast
array of antimicrobial peptides, the cysteine-rich peptides form a large group in which a certain disulfide motif stabilizes the molecular scaffold. They are all small (29–54 amino acid residues) and highly basic (pI > 10). Accordingly these plant antimicrobial peptides are usually classified by the number and pairing pattern of their disulfide bridges (Broekaert et al., 1990; Zasloff, 2002). So far a series of three-dimensional structures belonging to this group of antimicrobial peptides, including thionins (Orru et al., 1997), plant defensins (Segura et al., 1998), hevein-like (Martins et al., 1996; Nielsen et al., 1997) and knotin-like (Gao et al., 2001) peptides, have been reported. In these structures at most eight cysteines pairing into four disulfide bridges were found. Here we report the crystal structure of an antifungal protein, EAFP2, distinct with ten cysteines pairing into five disulfide bridges at an atomic resolution.

The Eucommia antifungal protein EAFP2 was lately identified from the tree Eucommia ulmoides Oliver (Gutta-parcha tree) (Huang et al., 2002), whose bark, called Du-Zhong in Chinese, is a traditional medicine used as a tonic for reducing blood pressure and contains a kind of latex similar to that of rubber tree. The sequence of EAFP2 consists of 41 residues with a N-terminal blockage by pyrogalylamyl acid. Its primary structure elucidated by tandem mass spectroscopy in combination with automated Edman degradation shows ten cysteines, which are cross-linked to form five disulfide bridges with a pairing pattern (C1–C5, C2–C9, C3–C6, C4–C7, and C8–C10) (Huang et al., 2002) (Fig. 1). This is the first finding of plant antifungal peptide with a five-disulfide motif. Besides, EAFP2 contains four positively charged arginines without any negatively charged residues. Bioassays showed that EAFP2 exhibited a relative broad antifungal spectrum against a series of pathogens from cotton, wheat, potato, tomato, and tobacco. The inhibitory activity can be effective on both cell wall chitin-containing and chitin-free fungi. The antifungal effects can be strongly antagonized by calcium ions (Huang et al., 2002). For all these properties in association with the peculiar primary structure, the molecular mechanism of the antifungal activity of EAFP2 becomes interesting. As the first step, the crystal structure of EAFP2 was determined. In this paper we will report the ab initio structure determination of EAFP2 at 0.84 Å resolution and its main structural features at the atomic precision. The structural relationship with other relative proteins and the possible structural basis for the functional properties of EAFP2 will also be discussed.

2. Materials and methods

2.1. Crystallization, data collection, and processing

Crystals of EAFP2 were obtained by the hanging-drop vapour-diffusion technique, with sodium acetate as the precipitant at pH 5.0–6.0 containing 5% (v/v) isopropanol, following a protocol previously described (Xiang et al., 2002).

X-ray diffraction data for EAFP2 were collected at 283 K from a single crystal at the BL-18B beamline of the Photon Factory in Tsukuba, Japan. A total of 190 frames were collected at a wavelength of 1.00 Å using an ADSC Q4 CCD detector.

The diffraction data were processed with the HKL suite of programs (Otwinowski and Minor, 1997). Results show that the crystal belongs to space group P2_1 with unit cell parameters a = 19.011, b = 23.178, c = 30.720 Å, β = 98.560°. Observations (75 722) were recorded and merged to give 22 113 unique reflections. According to the generally accepted Sheldrick’s criteria for an effective atomic resolution assessment (Reflections with I > 2σ(I) in the outmost shell exceed 50% of the theoretical reflections) (Sheldrick, 1990), the effective resolution of the diffraction data set reaches to 0.84 Å. The quite low Rmerge (5.1%) and high (I)/(σ(I)) (26.5) values over the whole resolution range and in the outermost shell (Rmerge = 14.3% and (I)/(σ(I)) = 8.5 in the resolution range 0.87–0.84 Å) indicate the good quality.

Fig. 1. Sequence alignments of EAFP2 with hevein, Pn-AMP, Ac-AMP, UDA, and WGA. The pairing pattern of the disulfide bonds is indicated at the top. The secondary structures of EAFP2 are indicated at the bottom. The conserved cysteines and the additional two cysteines unique in EAFP2 are highlighted in white on a gray and a black background, respectively; the conserved residues included in chitin binding are highlighted in box. Hevein, rubber latex hevein (Broekaert et al., 1990); Pn-AMP, antimicrobial peptide from Pharbitis nil (Koo et al., 1998); Ac-AMP, antimicrobial peptide from Amaranthus caudatus (Broekaert et al., 1992); UDA, Urtica dioica agglutinin (Beintema and Pemans, 1992); and WGA, wheat germ agglutinin (Wright et al., 1984). The figure was prepared with ALSCRIPT (Barton, 1993).
of the data. A summary of data collection and processing is given in Table 1.

2.2. Ab initio structure determination of EAFP2

The structure of EAFP2 was determined by ab initio direct methods using SnB version 2.1 (Weeks and Miller, 1999) with the 0.84 Å data set. The 1000 trials structures evaluated (4 days on an Intel P4 1.7 PC running Linux) showed the expected bimodal distribution. Twenty three of the 41 residues could be identified from the peaks in the initial E-map of the best solution.

The initial model building was started with a partial model constructed from the initial E-map of the best solution. The automatic model building procedure was used with the program ARP-wARP (Perrakis et al., 1997) and the results were out of our wildest expectations. More than 80% of the model could be correctly constructed by the program and almost all the backbone and side chain atoms could be clearly located in the result sigma-A weighted 2mF_o–DF_c electron-density map. The full initial model was finally completed with few efforts using the program O (Jones et al., 1991) and then used for the refinement.

2.3. Structural refinement

The model of EAFP2 was refined with SHELX97 (Sheldrick and Schneider, 1997) against experimental intensities. The refinement was carried out against 96% of the measured data. The remaining 4% (885 reflections) were randomly excluded from the data set and used as a cross-validation test using the free R factor (Brünger, 1992). The high resolution data were gradually included in the refinement. In the final steps of the refinement all data were included.

Atomic coordinates and isotropic displacement parameters were initially refined using conjugate gradient algorithm within the limited resolution from 3.0 to 1.5 Å. This isotropic refinement converged to an R factor of 13.44% and an R_free of 16.73% with a bulk solvent correction. The extension of resolution to 1.0 Å, with no changes of the model and B factors, led to a decrease of R_free but an increase of R factor. Residual densities around many of the protein atoms especially the heavier atoms such as sulphur in the difference map strongly suggested that anisotropic vibration should be accounted for at this time. The switch from isotropic to anisotropic displacement parameter refinement for all protein atoms resulted in a sharp drop of the R factor to 10.23% (ΔR ≈ 3.8%) with a comparable decrease of the free R to 11.74%. Many side chain atoms and one section of main-chain atoms of Ala32–Gly33 could be clearly identified to have multiple conformations now. Two acetate anions and some more water molecules were also identified and added to the model. After several rounds of model building and anisotropic refinements, all reflections were then included. The extension of resolution to its maximum, 0.84 Å, revealed more details of the structure. At this stage, some hydrogen atoms could be observed now even before they were generated as riding atoms and minor errors such as the misplaced N°2 and O°81 atoms in Gln38 were clearly revealed by the disagreements of their bond lengths to the target values. When hydrogen atoms were added to the model at calculated positions, R and R_free were reduced to 6.95 and 7.85%, respectively. A parallel job with the same model but excluding hydrogen atoms showed that approximately 1.25% of the drop in the R factors was contributed by the hydrogen atoms. Since then, attentions were paid to the solvent molecules. Majority of the water sites were assigned fixed occupancies of unit or less than unit according to their residue density in the F_o–F_c map. Solvent sites near the disordered residues were intuitively selected as partially occupied and their occupancies would be linked to the appropriate alternative conformations. Some restrains were also relaxed at this stage by setting a relatively large standard deviation values. After two rounds of adjustments, the last round of refinement was performed using all reflection data and a final R factor of 6.84% for 22 113 reflections was obtained. Statistics of the refinement are summarized in Table 2.

3. Results

3.1. Atomic resolution electron-density map and molecular model

The final electron-density map is of excellent quality and atomicity (see Fig. 2). All non-hydrogen protein
atoms including those with multi-conformations can fit to the articulated densities with one centre. Individual atoms such as C, N, and O can be distinguished on the basis of the volume of the density. Electron densities of 87% of the protein atoms are still visible even at a contouring level of 5.0σ (4.57 e/Å³). All dual conformations, including the side chains of Ser5, Arg9, Pro10, Ser25 and the main chain of residues 32–33, can be identified with definite densities, though, which are relatively weaker. In

the double conformations of residues 32–33, the alternate peptide groups are related each other by a rotation of about 180°, which is simply termed as a peptide flip (Fig. 2C). The electron densities at the N-terminal residue clearly reveal a five-atom ring (Fig. 2B), which was modelled as a L-pyroglutamyl acid residue with the geometric parameters from the Cambridge Structural Database (CSD) and put into the refinement as a non-regular residue. At the final stage of the refinement, no stereochemical or geometrical restraint was imposed on this residue. The final stereochemical parameters of this residue from the unrestrained refinement show good agreement with their target values and the accurate densities of this residue (Fig. 2B) confidently confirm a N-terminal blockage by pyroglutamyl acid as predicted from the sequence analysis (Huang et al., 2002).

The final refined EAFP2 model consists of a total of 305 protein atoms, 64 water molecules, two acetate anions, and 280 protein H atoms built according to the geometrical criteria. Four side chains belonging to Ser5, Arg9, Pro10, and Ser25 and one section of the main chain of residues 32–33 were modelled in double conformations with confidence. The analysis of the stereochemistry of the EAFP2 model with the program PROCHECK (Laskowski et al., 1993) shows that all geometric parameters are within the limits expected for this resolution. Compared to their target values, the final model of EAFP2 has an average deviation of 0.017 Å and 1.74° for bond lengths and bond angles, respectively. Analysis of the main-chain conformations

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* Values in parentheses are the number of atoms with occupancies less than unit.

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Fig. 2. Representative electron densities for the EAFP2 structure at ultrahigh resolution. Carbon, nitrogen, oxygen, and hydrogen atoms in the model are coloured in black, blue, red, and green, respectively. (A) View of the β-sheet region of the final model superimposed on 3Fo – 2Fc electron-density map contoured at 3.0σ (cyan) and Fo – Fc hydrogen-omit density map contoured at 2.5σ (red). (B and C) 3Fo – 2Fc electron-density maps (contoured at 2.0σ and 1.5σ, respectively) around the N-terminal non-regular pyroglutamyl acid residue (Pca1) (B) and the main chain 32–33 with double conformations (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)
shows that a total of 90% of the residues falls in the most favoured regions of Ramachandran plot and no residues are found in the disallowed or generously allowed regions. The stereochemical statistics of the final model are summarized in Table 2.

The errors of the parameters in the refined model are also estimated only from the diffraction data without reference to their mean values. To get it, the final model was further refined with SHELX/H in full matrix least squares mode (Sheldrick and Schneider, 1997; Stout and Jensen, 1989). The mean errors in bond lengths and bond angles evaluated in this way are 0.016 Å and 1.07°, respectively. It is noticed that where the structures modelled as multi-conformations have rather high estimated errors. When the multi-conformation parts are excluded from the statistics, the mean errors in bond lengths and bond angles evaluated are 0.011 Å and 0.66°, respectively.

3.2. Anisotropic displacement

Anisotropic temperature factors were assigned to all non-hydrogen atoms in the EAFP2 model including solvent molecules. The drop of about 4.8% in R factor and 3.8% in R_free after anisotropic refinement confirms that EAFP2 is better described in terms of anisotropic displacement parameters. The average equivalent B factors calculated after the anisotropic refinement are 4.921 and 7.246 Å² for main-chain and side-chain atoms, respectively, which is slightly larger than the average B factor of the isotropic model. Similar to the results from other proteins refined at atomic resolution, the anisotropic refinement does not result in a large shift of the atomic position and the r.m.s.d.¹ for the Cα atoms between the models before and after the anisotropic refinement is only 0.022 Å.

The mean anisotropy factor (minimum eigenvalue divided by maximum eigenvalue) is equal to 0.48 and 0.39 for main-chain and side-chain atoms of EAFP2, respectively. This means that all ellipsoids approximating the thermal motion of atoms are twice as large in one direction as in other. The distribution of the anisotropy factors for all protein atoms is approximately symmetric around the mean value of 0.44 with a standard deviation of 0.14. These values agree very well with the result obtained by Merritt (1999) on an analysis of anisotropic refined atomic structures in the Protein Data Bank. The thermal motion is smaller and less anisotropic in the core region than in the surface region and this is consistent with the vision inspection of the ORTEP-III (Burnett and Johnson, 1996) drawing of all C, Cα, and N atoms and the five disulfide bonds (Fig. 3). Large anisotropic motions (anisotropy factor less than 0.2) are observed in residues Thr2, Ser5, Ala13, Tyr20, Ser25, Gly33, Asn34, and Cys39 at the surface of the molecule. Compared to the protein atoms, the solvent atoms are rather anisotropic. They have a mean anisotropy factor of 0.28 with a standard deviation of 0.16. For different type of atoms, it is worth to note that the average anisotropy factor of the sulphur atoms (0.431) is smaller than those of the nitrogen (0.479) and carbon (0.442) atoms.

3.3. Hydrogen atoms and hydrogen bonds

The precise electron-density map at the atomic resolution allows an analysis of the occurrence of visible hydrogen atoms in the electron density. Fig. 2A shows part of an omit map at the three-stranded β-sheet, in which the positions of hydrogen atoms around the main chain Cα or N atoms can be clearly observed as positive peaks. Many side-chain hydrogen atoms can also be observed in the electron density, such as the side chain of Tyr21, in which all positions of the hydrogen atoms are clearly visible (Fig. 2A). Using a

Abbreviations used: r.m.s.d., root-mean-square deviation; SYM, symmetry related molecule.
criterion that the presence of electron density peaks within 0.3 Å from calculated positions of hydrogen atoms, more than 65% of the hydrogen atoms (182/280) in protein are observed in the difference map at a contour level of 1.5σ (0.12 e Å\(^{-3}\)), while almost all of the main-chain hydrogen atoms can be seen at a contour level of 2.5σ (0.20 e Å\(^{-3}\)), except the region adopting multiple conformations.

A total of 31 intramolecular hydrogen bonds are found in the final model. Twenty two of them are main-chain N–H···O interactions. Based on the calculated positions and the default geometric parameters for riding hydrogen atoms implemented in SHELX97, the average H···O and N···O distances and the N–H···O angle of these main-chain hydrogen bonds are 2.21, 3.00, and 153.6°, respectively. The rest 9 hydrogen bonds are side-chain–main-chain interactions with the average H···A (acceptor) and D (donor)···A distances of 2.31 and 3.09 Å, respectively, and the D–H···A angle of 153.8°. The shorter mean distance of these hydrogen bonds suggests that they are stronger interactions and important for maintaining the overall fold of the molecule. The clear visible main-chain hydrogen positions also allow an analysis of C\(^\alpha\)-H···O interactions in EAFP2 of the 27 interactions with H\(^\alpha\)···O distances shorter than 2.7 Å, only 3 contacts fit the criteria of this kind of weak hydrogen bonds.

### 3.4. Intermolecular contacts

EAFP2 crystals are extremely dense (\(V_0 = 1.61 \text{ Å}^3/\text{Da}\)). Packing analysis shows that the molecule packs against many neighboring molecules with complement surfaces. A total of 12 symmetry related molecules can be found within a distance of 5 Å from the molecule. Using a probe radius of 1.4 Å, the total buried molecular surfaces between the individual molecule and the symmetry related molecules calculated with CNS (Brünger et al., 1998) are 1749 Å\(^2\) (the surface areas buried by each lattice contact are calculated as one half of the difference between the total surface areas of two non-contacting molecules, and of two molecules related by the specified symmetry), while the total solvent accessible area of the molecule is 2573 Å\(^2\). There are 8 direct protein–protein hydrogen bonds in between these buried surface areas, including 5 O···3.4 N\(^{\delta2}\) (SYM) 2.95 Å, 5 O···3.6 N\(^{\delta1}\) (SYM) 3.11 Å, 12 O···3.40 N\(^{\delta2}\) (SYM) 2.90 Å, 12 O···25 N (SYM) 3.00 Å, 12 N\(^{\delta2}\)···25 O (SYM) 2.88 Å, 22 O···3.6 O (SYM) 2.77 Å, 27 N···41 O (SYM) 3.29 Å, and 27 N···40 O (SYM) 3.07 Å. Furthermore, there is a salt bridge mediated by an ordered acetate anion, which is formed between Arg6 and Arg40 of the neighboring molecule with the acetate anion just between the two arginines.

### 3.5. Structural features of EAFP2

#### 3.5.1. General structure

The general fold of EAFP2 is depicted in Fig. 4. It contains some short secondary structure elements, including two stretches of helix formed by residues 3–6 (H1) and 27–31 (H2) and a three-stranded antiparallel β-sheet formed by residues 16–18 (β1), 23–25 (β2), and 35–37 (β3) (Fig. 1). Though with very short length in each strand, the β-sheet is the structural core of the molecule. H1 running from residues 3–6 is in fact one turn of a 3\(_{10}\) helix, while H2 is a regular α-helix. The two helices pack with one side onto the opposite sides of the β-sheet and run approximately 60 and 30° relative to the β-sheet, respectively (Fig. 4). Six β-turns are found in connection of these secondary structures, which belong to four types of β-turns, I, II, VIII, and VIa, respectively (Hutchinson and Thornton, 1994). The unusual type VIa turn is located at position Arg6–Arg9 within the N-terminal part of the structure, in which a proline residue, Pro8, occupies position \(i + 2\) and the peptide bond between Cys7 and Pro8 adopts a cis configuration with the \(\omega\) angle of 17.6°. The type VIa β-turns are usually involved in a double turn which consists of the type VIa turn followed by a type IV turn (Hutchinson and Thornton, 1994). Here the VIa type turn is also involved in a double turn but followed by a type VIII turn (residues 7–10). Position \(i + 3\) of the VIII turn is occupied by another proline residue, Pro10, while no cis peptide bond is found here. Other four β-turns (residues 12–15, 19–22, 31–34, and 38–41) possess the regular conformations. Among them, the turn formed by residues 31–34 is of high flexibility, in which segment 32–33 has been
ambiguously identified with double conformations as described above, while the successive alanine and glycine residues in it (Fig. 1) may be the reason of the dual conformations found here.

3.5.2. The distinct five-disulfide-bond motif

The striking feature of EAFP2 distinct from other plant antifungal peptides is that there are five disulfide bonds in such a small 41-residue peptide. The precise density map definitely showed that the disulfide bonds took the pairing pattern of Cys3–Cys17, Cys7–Cys37, Cys11–Cys23, Cys16–Cys30, and Cys35–Cys39 (C1–C5, C2–C9, C3–C6, C4–C7, and C8–C10) (S1–S5 in Fig. 4). Compared to the relative antifungal peptides with four disulfide bonds such as the hevein-like peptides, the disulfide bonds Cys7–Cys37 (S2 in Fig. 4) is distinct, which connects the N-terminal ten residues to the C-terminal segment to give rise to the tertiary correlation between these two parts (Figs. 5 and 6). Except Cys3–Cys17, which is almost completely covered by H1, the other four disulfide bonds are all solvent assessable. The exposure of the disulfide bonds may explain why the sulphur atoms have a smaller anisotropy factor. Disulfide bonds Cys3–Cys17, Cys7–Cys37, and Cys11–Cys23 connect the N terminal segment 1–12 to strands β1, β3, and β2 of the β-sheet, respectively. While the other two disulfide bonds Cys16–Cys30 and Cys35–Cys39 connect β1 to H2 and β3 to the turn Gly31–Asn34, respectively (Fig. 4). None of the disulfide bonds was found in connection of different strands of the Β-sheet. The five disulfide bonds creates a compact and rigid structure which makes EAFP2 extremely stable. Actually, the bioassay has showed that the antifungal activities of EAFP2 remain unchanged even in boiling water for 30 min (Huang et al., 2002).

3.5.3. Main structure feature

Though EAFP2 structure adopts a compact fold, its tertiary arrangement is subtle. The molecule could be divided into two sectors, A and B, along the disulfide bond Cys3–Cys17 (S1) and the reverse turn 31–34 as shown in Fig. 5. The sector A consists of the N-terminal ten residues and the C-terminal segment 35–41, which are cross-linked by the disulfide bridges Cys7–Cys37 (S2) and Cys35–Cys39 (S5). It features a cationic surface consisting of all four positively charged residues, Arg6, 9, 36, and 40, of the molecule (see Fig. 5). In this tertiary organization, the unique disulfide bridge Cys7–Cys37 (S2) plays a critical role. Actually, in the hevein-like peptides or domains, such as hevein, which contain four disulfide bonds without that one corresponding to Cys7–Cys37 (S2) of EAFP2, the N- and C-terminal segments are rather free in orientations and adopt quite different conformations. In EAFP2, the insertion of the disulfide bond Cys7–Cys37 (S2) in between N- and C-terminal segments connects these two segments and brings the neighbouring four arginines onto an external surface to form the cationic surface (Fig. 5). It is worth to note that residues 6–10 around Cys7 are successively involved in an unusual VIa–VIII double turn, in which the peptide bond Cys7–Pro8 adopts a cis configuration as described above. These particular structural elements may be favourable to the specific orientation of Cys7 in bonding with Cys37.

The sector B of EAFP2 molecule, roughly opposite to sector A, comprises 20 residues from Cys11 to Cys30, which are cross-linked by disulfide bridges Cys11–Cys23 (S3) and Cys16–Cys30 (S4). This small domain features a hydrophobic surface containing a cluster of hydrophobic and aromatic residues of Leu15, Ile19, Tyr 20, Tyr22, and Tyr29. Among others, three aromatic residues (Tyr20, 22, and 29) in association with a serine residue (Ser18) are noticeably existed on this surface (Fig. 5). These residues are known critical for chitin binding in all chitin-binding hevein-like proteins, such as hevein, Ac-AMP, and UDA. The general conformation of sector B is also similar to the corresponding parts of the hevein-like proteins (Fig. 6).

The specific tertiary arrangement produces a cationic surface and a hydrophobic surface, which makes EAFP2 molecule possess the amphiphilic character.
These structural features should be related to the functional properties of the antifungal peptide EAFP2. It is noteworthy that the rather flexible turn 31–34 and the peptide flip of residues 32–33 in between sector A and B may induce certain relative mobility in solution, which might also play a role in the functional performance of EAFP2.

4. Discussion

4.1. Structural relations with the hevein-like proteins

The sequence of EAFP2 shows certain homologies to a specific group of antifungal peptides, the hevein-like peptides such as hevein from Hevea brasiliensis (rubber tree) (39%), Ac-AMP from Amaranthus caudatus seeds (39%) and Pn-AMP from the seeds of Pharbitis nil (51%) (Huang et al., 2002) (Fig. 1). The hevein-like peptides usually consist of 30–43 amino acid residues cross-linked by 3 or 4 disulfide bonds and feature a chitin-binding site comprising three aromatic residues and one serine residue. This type of structure is also found in a variety of plant proteins, such as wheat germ agglutinin (WGA) and Urtica dioica agglutinin (UDA) as single or multiple copies referred as hevein domain (Beintema and Peumans, 1992; Wright et al., 1984). Their antifungal effects are assumed to be associated with their specific chitin-binding activities (Broekaert et al., 1989; Raikhel et al., 1993).

The three-dimensional structures of several hevein-like proteins have been studied, including NMR structures of hevein (Andersen et al., 1993) and Ac-AMP (Martins et al., 1996), crystal structures of WGA (Wright, 1977, 1987), a lectin comprising two monomers with four hevein domains each and UDA (Harata and Muraki, 2000; Saul et al., 2000), a lectin with two hevein domains.

Structure comparisons show that the sector B consisting of residues Cys11 to Cys30 of EAFP2 can be well superimposed with the corresponding segments in all hevein-like proteins (Fig. 6). The r.m.s.d. for equivalent superimposed with the corresponding segments in all consisting of residues Cys11 to Cys30 of EAFP2 can be well

The most striking structural diversity of EAFP2 from hevein and the hevein-like proteins appeared at the sector A consisting of the N-terminal ten residues and C-terminal segment 35–41 (Figs. 5 and 6). In the relative structures reported so far, the N- and C-terminal segments corresponding to the sector A are rather free and adopt quite different conformations because there is no tertiary restraints in between. There are also no concentrated cationic residues in the sequences of this region (Fig. 1). In EAFP2, all four cationic residues, Arg6, 9, 36, and 40 just occur in these two segments, which are brought together by the unique disulfide bridge Cys7–Cys37 (S2) to form a cationic surface as described above (Fig. 5). There is an additional salt bridge between Arg9 and the C-terminal carboxyl group, which may also contribute to the stabilization of this local structure. Moreover, the N-terminal residues 3–6 form a 3_10 helix and the residues Arg6 to Pro10 adopt a peculiar double turns of VIa–VIII type with a cis configuration of the peptide bond Cys7-Pro8 in EAFP2, which are not found in hevein and the hevein-like proteins.

4.2. Structure–function relationship

4.2.1. The chitin-binding site and the inhibitory effects on cell wall chitin-containing fungi

EAFP2 exhibits chitin-binding activity and inhibitory effects on the growth of cell wall chitin-containing and chitin-free fungi (Huang et al., 2002), which is distinct from the hevein-like proteins reported so far. The fine three-dimensional structure reveals the possible structural basis of its functional properties.

The protein–carbohydrate interaction studies of the hevein-like proteins either by NMR or X-ray crystallography (Asensio et al., 1998; Harata and Muraki, 2000; Saul et al., 2000; Wright, 1992) revealed that they possess the common chitin-binding site constituted by three conserved aromatic residues and one serine residue. The antifungal activities of these peptides are assumed to be associated with their specific chitin-binding activities since chitin is the main structural component of fungal cell wall (Broekaert et al., 1989; Raikhel et al., 1993). Polysaccharide binding assay showed that EAFP2, like the hevein-like proteins, could bind crab shell chitin specifically (Huang et al., 2002). The structural comparisons, as described above, identified that the structure of the sector B (residue 11–30) is similar to the chitin-binding domain in the hevein-like proteins, in which residues Ser18, Tyr20, Tyr22, and Tyr29 corresponding to the chitin-binding site adopts the conformations almost identical to those of the hevein-like proteins (Fig. 6). Therefore, it is plausible to suppose
that the inhibitory effects of EAFP2 on chitin-containing fungi are mostly related to the structural sector B through the chitin-binding mechanism as suggested in the hevein-like peptides.

The fine structure of EAFP2 revealed the precise stereochemistry of the chitin-binding site. The three tyrosine residues of the chitin-binding site are all exposed to solvent and so form a relative hydrophobic surface pocket around the key residue Ser18 (Fig. 5). The side chain atom O\(^\prime\) of Ser18 in EAFP2 is hydrogen bonded to the main chain N atoms of Tyr20 and Tyr22. These two hydrogen bonds obviously decrease the thermal motion of the side chain of Ser18 and fix it to a certain orientation. Ser18 is the only serine residue without multiple conformations in the molecule, while the other two serine residues, Ser5 and Ser25, are unambiguously identified with dual conformations. In addition to the two hydrogen bonds with the protein atoms, O\(^\prime\) of Ser18 is also hydrogen bonded to a water molecule, which is just located in the hydrophobic pocket. In the \(F_o - F_c\) hydrogen-omit density map (Fig. 7), the orientation of the hydrogen atom at C\(^\alpha\) of Gly24 clearly directs to the centre of the Tyr29 ring, showing a C–H–π interaction definitely. The perpendicular distance of the C\(^\alpha\) atom of Gly24 to the plane of the ring is 3.29 Å. Delta-matrix of the anisotropic displacement parameters between the three tyrosine residues suggests that the aromatic ring beyond the C\(^\beta\) atom of each tyrosine residue may behave as a rigid body. While significant differences between the different two tyrosine residues clearly reveal the different vibration modes of the three tyrosine residues (Fig. 8). Further anisotropic displacement analyses using the TLS model (Schomaker and Trueblood, 1968) with THMA14 (Trueblood and Schomaker, unpublished results) indicate that the dominant rotation of the side chain atoms of these tyrosine residues is around the C\(^\delta\)–C\(^\gamma\) bonds, ranging from 6.2° for Tyr29 to 16.2° for Tyr20. The estimated translation amplitudes indicate near-isotropic vibrations of about 0.2 Å for Tyr29 and 0.3 Å for Tyr20 and Tyr22. Comparison of the thermal motions of the three Tyr residues shows that Tyr29 is of smaller thermal motion due to its less exposure and the strong C–H–π interaction with Gly24. In the sequence alignments of EAFP2 and the hevein-like proteins, the aromatic residue type at position 29 is almost invariant as a tyrosine residue (Fig. 1). The small thermal motions (Fig. 8) and invariance of Ser18 and Tyr29 revealed by the EAFP2 structure must be of functional significance. Actually, these two residues are all directly hydrogen-bonded to the sugar in the complex structures of protein and carbohydrate (Harata and Muraki, 2000; Saul et al., 2000). Therefore, it is plausible to infer that their small thermal motions may be responsible for precisely anchoring of the ligand during the chitin recognition process.

4.3. Possible structural basis for the inhibitory effects on cell wall chitin-free fungi

In the last decade, a variety of antifungal peptides have been discovered and a series of their three-dimensional structures have been determined either by X-ray crystallography or NMR analysis. Though these structures showed a wide diversity from the amphiphilic helical structure to the α-helix–β-sheet motif, etc., (Aumelas et al., 1996; Hwang and Vogel, 1998; Zasloff, 2002), most of them have an amphiphilic surface in which clusters of cationic and hydrophobic residues are spatially organized in discrete sectors of the molecule. It has been suggested that the cationic sector may be generally critical for the antimicrobial activity through binding to the negatively charged phospholipid of microbial cell membrane (Hwang and Vogel, 1998). Such interactions could play roles in the initial binding of antimicrobial peptides with microbial cell membrane to disturb the normal membrane functions. In EAFP2 a cationic surface consisting of four positive charge-potential residues, Arg6, 9, 36, and 40, is protuberantly occurred at the structural sector A (Fig. 5), which makes the EAFP2 molecule possess the amphiphilic character.
in the tertiary arrangement (Fig. 5). Referring the knowledge on antimicrobial peptides mentioned above, this cationic surface on the sector A of EAFP2 may play essential roles in inhibiting the growth of cell wall chitin-free fungi. Actually, the bioassay showed that the antifungal effects of EAFP2 could be strongly antagonized by calcium ion (Huang et al., 2002), which identified the dependence of the antifungal activities of EAFP2 on the electrostatic interaction. Most probably the hydrophobic surface containing the chitin-binding site on the structural sector B of EAFP2 (Fig. 5) also contribute to the performance of the inhibitory activity on chitin-free fungi through non-specific hydrophobic interactions.

Therefore, for chitin-free fungi EAFP2 may take the same amphiphilic approach as describe above. The distinct disulfide bond Cys7–Cys37 seems critical to give the EAFP2 molecule an amphiphilic design.

Protein Data bank accession code. The coordinates and structure factors of the EAFP2 structure have been deposited to the PDB with accession number of 1P9G (RCSB019174).

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