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Solution structure of a defense peptide from wheat with a 10-cysteine motif

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ABSTRACT

Hevein, a well-studied lectin from the rubber tree *Hevea brasiliensis*, is the title representative of a broad family of chitin-binding polypeptides. WAMP-1a, a peptide isolated from the wheat *Triticum kiharae*, shares considerable similarity with hevein. The peptide possesses antifungal, antibacterial activity and is thought to play an important role in the defense system of wheat. Importantly, it features a substitution of the conserved serine residue to glycine reducing its carbohydrate-binding capacity. We used NMR spectroscopy to derive the spatial structure of WAMP-1a in aqueous solution. Notably, the mutation was found to strengthen amphiphilicity of the molecule, associated with its mode of action, an indication of the hevein domain multi-functionality. Both primary and tertiary structure of WAMP-1a suggest its evolutionary origin from the hevein domain of plant chitinases.

1. Introduction

Plants have developed both constitutive and inducible resistance mechanisms against pathogens. Defensive weapons include morphological barriers, secondary metabolites (e.g. phytoalexins and phytoanticipins), and defense (antimicrobial) proteins and peptides (AMPs). AMPs are classified into several families, including thionins, defensins, lipid-transfer proteins, knottin- and hevein-like peptides [1,2]. Hevein from the rubber tree *Hevea brasiliensis* is the title peptide of the latter group, characterized by a distinct fold and a number of conserved amino acid residues (cysteines form disulfide bridges providing structural stability, and three aromatic residues with two glycines and a serine constitute the so-called chitin-binding motif implicated in carbohydrate recognition and believed to underlie the biological activity) [3–5].

Recently we have isolated AMPs WAMP-1a and -1b (differing by an additional C-terminal arginine) from seeds of the wheat *Triticum kiharae* Dorof. et Migusch. [6]. These peptides exhibit similarity with hevein-type peptides and chitin-binding domains of plant class I chitinases (Fig. 1). Moreover, WAMPs possess 10 cysteines involved in five S–S–bridges, located exactly as in chitinases. Other hevein-type peptides, namely EAFPs from *Eucommia ulmoides* [7] and Ee-CBPs from *Euonymus europaeus* [8], also contain 10 Cys residues, but their cysteine motifs and disulfide connectivity patterns differ from that of chitinases.

It was proposed that antifungal activity of WAMPs arises from their chitin-binding capacity. The peptides were also found active against several strains of Gram-positive and Gram-negative bacteria [6]. To address the structural basis of these activities, we have undertaken investigation of WAMP-1a in the present work. The peptide structure in aqueous solution was studied by NMR spectroscopy. The obtained results shed light on the structure–activity relationships in hevein-type peptides and multi-functionality of the hevein fold, and provide important clues to the evolutionary origin of WAMPs.

2. Materials and methods

To provide sufficient material for the investigations, recombinant WAMP-1a was produced in a prokaryotic expression system as described [6,9]. The peptide sample in aqueous solution was prepared by dissolving 0.5 mg of WAMP-1a in 250 μL of $H_2O/^2H_2O$ (9:1). Nearly complete ^{13}C,^{15}N,^1H-assignments of WAMP-1a were obtained at natural abundance, using [^{15}N,^1H]-, [^{13}C–^1H]-HSQC, [^{13}C–^1H]-HMBC, [^{13}C–^1H]-HSQC-TOCSY spectra (in preparation). Calculation of chemical shift index (CSI) and random coil index (RCI) values was performed as described [10,11]. All two-dimensional NMR spectra were recorded with an Avance-700 spectrometer (Bruker, Germany) fitted with a cryoprobe. The

Abbreviations: AMPs, antimicrobial peptides; ITC, isothermal titration calorimetry; CSI, chemical shift index; MHP, molecular hydrophobicity potential; RCI, random coil index; WAMP-1a, 44-amino-acid-residue-long wheat antimicrobial peptide with the amino acid sequence: AQRCGDQARGAKCPNCLCCGKYGFCGSGDAY CGAGSCQSQCRGC.

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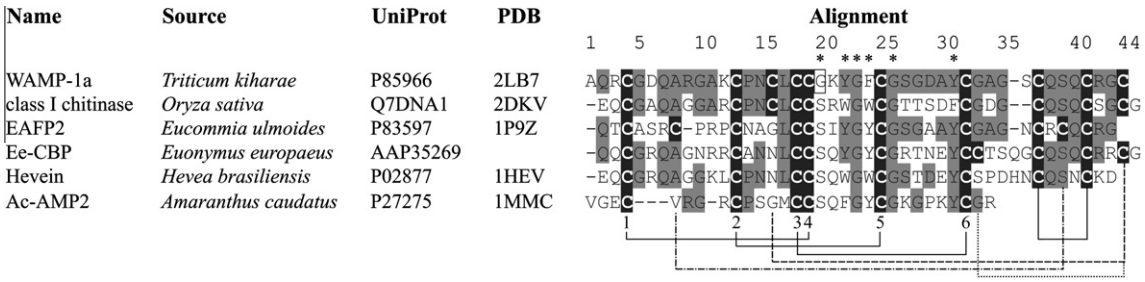

Fig. 1. Sequence alignment of WAMP-1a with selected hevein-type AMPs and chitin-binding domain of a class I chitinase from rice. Numbering is according to WAMP-1a. Cysteine residues are shaded in black; other residues identical to those in WAMP-1a are shaded in gray. Six conserved cysteines are numbered below; residues of the carbohydrate-binding site are marked with asterisks; Gly-20, replacing the conventional serine in WAMP-1a is boxed. Disulfide connectivities are shown below, S–S–bonds found in hevein are presented by solid lines, the additional fifth disulfide is shown by dashed line in WAMP-1a and chitinase, dashed-dotted line in EAFP2, and dotted line in Ee-CBP.

spectra were acquired for WAMP-1a dissolved in either H_2O, or ^2H_2O. The temperature and pH of the sample were varied in the range 10–45° C and 3.5–7.5. Water suppression and spectra processing were carried out as described earlier [12]. For measurements of the deuterium exchange rates TOCSY spectra (mixing time of 80 ms) were acquired every 20 min during 24 h. All peak volumes in NOESY spectra recorded in H_2O were calibrated, assuming 2.5 Å distance between H_iN and $H_{i-1}\alpha$ atoms in β-strands. Non-overlapping cross-peaks were selected for the calibration procedure. Scalar spin–spin $^3J_{\alpha\beta}$ constants and stereospecific assignments of the methylene protons were determined from DQF-COSY spectra. The spin–spin $^3J_{\alpha N}$ constants were measured from one-dimensional spectra acquired in the temperature interval of 10–45° C. Flexible side chains were not constrained to prevent distortion of the structure. NOE, hydrogen–deuterium exchange data, and the values of $^3J_{\alpha N}$ coupling constants were used to determine the secondary structure. H-bond constraints were introduced at the final stage of the structure calculation with the following lower/upper values: 1.7/2.2 Å for HN–O, 2.6/3.5 Å for HN–C′, and 2.6/3.3 Å for N–O, respectively. Structure calculation was performed in several steps. At the first stage, dihedral angle and NOE distance restraints, originated from unambiguously assigned protons, were used. At the next stage, hydrogen bond restraints were added, if supported by NOEs, H–D exchange data, and low absolute values of amide proton temperature coefficients. In the final cycle, the S–S–bond constraints were added. Survey of the constraints is presented (Supplementary Table S1). The best 20 structures with lowest target function values were used for analysis of input constraints violation. Analysis of the structure and preparation of the images were performed with the MOLMOL [13] and VMD [14] programs. Molecular hydrophobicity potential (MHP) was calculated using the PLATINUM web-server [15]. Quality of the structures was assessed with the PROCHECK-NMR program [16]. Sequence similarity searches were performed using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was built with the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and refined manually. 3D structure comparison was performed using the Dali server (http://ekhidna.biocenter.helsinki.fi/dali_server/) [17].

3. Results

We used the full set of ^{13}C,^{15}N,^1H-chemical shifts to assess WAMP-1a dynamics via calculation of the RCI values and model-free order parameters (Fig. 2). These values suggest that the peptide is rather rigid on the RCI time-scale, in accordance with NOE-based analysis of WAMP-1a spatial structure (see below). This observation is valid for the whole set of temperatures (10–45° C) and pH (3.5–7.5) investigated.

Sequence-specific assignment of ^1H-NMR resonances was performed using the common strategy [18], which could be performed via observation of $d_{\alpha N}$ and/or d_{NN} NOEs ($d_{\alpha N}$ for Pro-14–Asn-15). According to the described protocol for determination of cis/trans configuration of X-Pro bond [19], trans-configuration of the Cys-13-Pro-14 bond was deduced from NOE between α-proton of Cys-13 and δ-protons of Pro-14. The full set of NOEs, $^3J_{\alpha N}$ coupling constants, slowly exchanging amide protons is presented (Supplementary Figure S1). The pairing scheme between cysteines in WAMP-1a was deduced upon homology considerations. This scheme (Fig. 1) was found not contradicting the observed NOEs.

Structural statistics for the calculated ensemble shows that the structure of WAMP-1a is well defined by the NMR data (Supplementary Table S2). The obtained structure is represented by an antiparallel four-stranded β-sheet comprising residues 2–3 (strand 1), 18–20 (strand 2), 24–26 (strand 3), and 36–39 (strand 4), a 3_{10} helix, residues 6–8, and an α-helix, residues 29–32 (Fig. 3A and B). WAMP-1a molecule is rather compact. Its fold is stabilized by five disulfide bonds and an array of as many as 20 hydrogen bonds. Configuration of Pro-14 and all of the disulfide bonds is well-defined (Supplementary Table S2). The peptide "core" is formed by the disulfides and Ala-8, Ala-11, Pro-13, and Leu-17 side chains. Amphiphilic properties of WAMP-1a are clear from the MHP [20] drawn on the surface of the molecule (Fig. 3C). The hydrophobic cluster (~360 Å^2) is formed by the side chains of aromatic (Tyr-22, Phe-24, Tyr-31) and aliphatic (Ala-1, Ala-30) residues (the hydrophilic surface amounts to ~1920 Å^2).

4. Discussion

4.1. WAMP-1a is a hevein-type plant defense peptide

The global fold of disulfide-rich peptides is recognized to be stabilized primarily by formation of disulfide bonds, and to a lesser extent, secondary structure and hydrophobic contacts [21]. Hevein and related peptides have the knottin-like topology characterized by two adjacent disulfide bonds (C1–C4 and C2–C5; Fig. 1), which are roughly perpendicular, i.e. form a cross in the so-called knottin-like core (Fig. 3). This motif has been suggested as a folding nucleus, conferring an evolutionary advantage to the proteins that contain it.

Sequence of WAMP-1a is similar to that of hevein-type peptides (Fig. 1); it also contains most of the conserved residues of the hevein chitin-binding motif (see below). Hevein-type peptides contain 3–5 disulfides, of which three are strictly conserved. Two S–S–bonds contribute to the formation of the knottin-like core, and the overall fold contains at least two strands of antiparallel β-structure (residues 18–20 and 24–26 in WAMP-1a) and a short α-helical turn (29–32). WAMP-1a

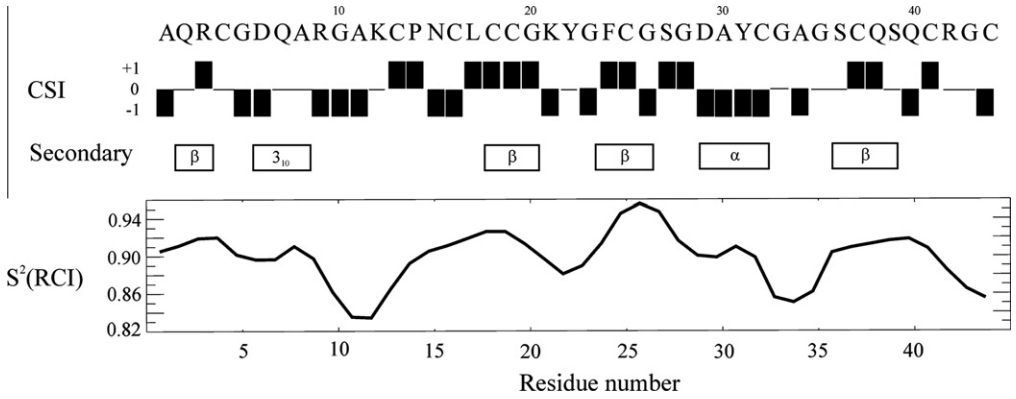

Fig. 2. Chemical shift-based data for WAMP-1a. Shown are: the CSI, secondary structure, and model-free order parameter (S^2), calculated from the RCI.

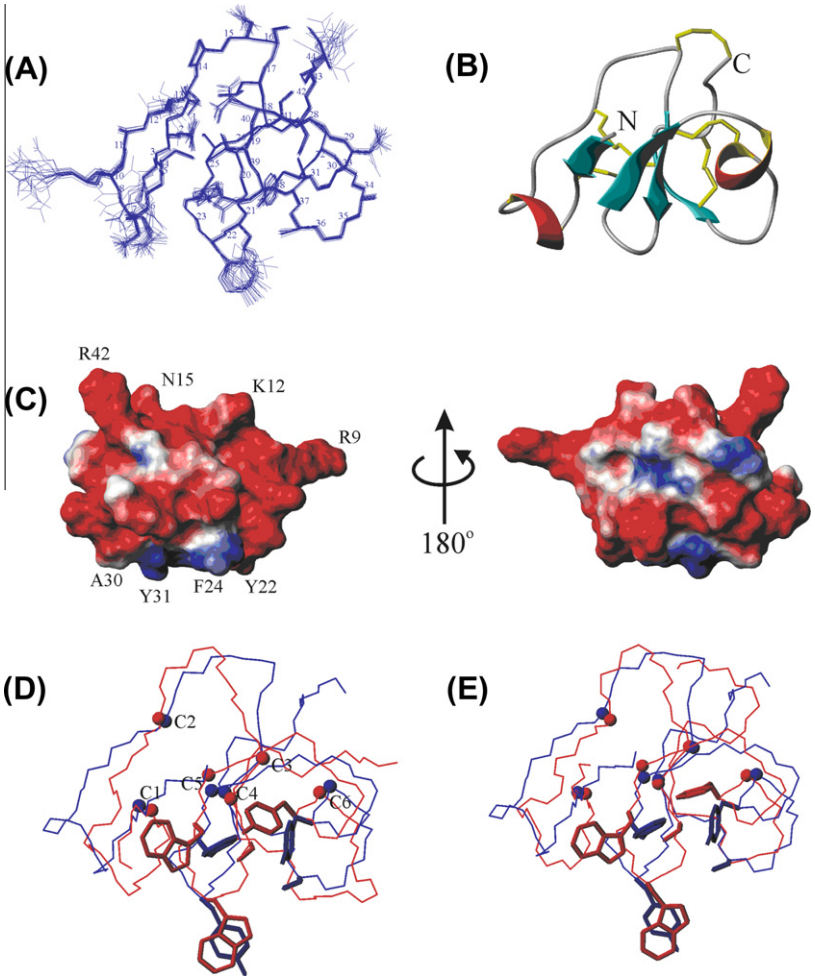

Fig. 3. Spatial structure of WAMP-1a and its comparison with hevein and chitin-binding domain of a class I chitinase. (A) Ensemble of 20 "best" structures, superimposed over backbone atoms. Residue numbers are indicated next to Cα atoms. (B) Ribbon representation of the structure No. 1 from the set, taken in the same orientation as in panel (A). β-Strands are shown with blue arrows (residues 2–3, 18–20, 24–26, 36–39), the 3_{10}-helix (residues 6–8) and α-helix (29–32) are shown as red-yellow ribbons. The disulfide bonds (between Cys residues 4–19, 13–25, 16–44, 18–32, and 36–41) are shown as yellow tubes. The N- and C-termini are marked. (C) MHP painted on the peptide surface. The red and blue areas correspond to polar and hydrophobic regions, respectively; residues at the periphery are marked. (D and E) WAMP-1a (blue) and hevein (in D) or rice chitinase hevein domain (in E) (red) superimposed over the Cα atoms of the conserved cysteines (shown with spheres). Side chains of the aromatic residues and serine forming the chitin-binding site in hevein and chitinase, and the corresponding residues in WAMP-1a (Tyr-22, Phe-24, Tyr-31), and Ser-36, are shown with thick lines (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

shares common properties of this fold, and similar spatial structures were reported for several chitin-binding hevein-type AMPs, such as hevein itself [4], EAFP2 from *E. ulmoides* [22] and Ac-AMP2 from *Amaranthus caudatus* [23], and the chitin-binding hevein domain of plant class I chitinase from rice [24] (Figs. 1 and 3). Taking into consideration the anti-pathogen activity of WAMP-1a, we conclude that it classifies as a hevein-type plant defense peptide.

4.2. WAMP-1a structure suggests its mechanism of action

Hevein-type AMPs studied to date are characterized by ability to bind chitin, which is associated with their biological activity, since chitin represents one of the major components in fungal cell walls. Peptide–carbohydrate interactions are determined primarily by the residues located close to the knottin-like core and forming the so-called chitin-binding site. In hevein, the aromatic residues Trp-21, Trp-23, and Tyr-30 are known to stabilize its complexes with N-acetyl-glucosamine oligosaccharides by means of van-der-Waals and CH-π interactions, whereas the hydroxyl group of the Ser-19 side chain forms a hydrogen bond with the carbonyl group of the acetamide moiety [5,25]. Replacement of this crucial serine results in significant decrease of the binding affinity [26]. Superimposition of the structures of WAMP-1a, hevein and chitinase chitin-binding domain is shown in Fig. 3D and E. In WAMP-1a a glycine residue (Gly-20) is present in the respective position of Ser-19 in hevein. An additional Ser-36 is present in WAMP-1a sequence, but is located away from the chtin-binding site. As a result, the WAMP-1a-carbohydrate complex cannot be stabilized by the above mentioned hydrogen bond. According to ITC, WAMP-1a does not bind to penta-*N*-acetylchitopentaose (V.A. Mitkevich, personal communication), as opposed to hevein exhibiting a dissociation constant in the micromolar range [5]. However, earlier, binding of WAMP-1a to contiguous polymers of chitin was demonstrated [6].

In some cases, antibacterial and antifungal activities of AMPs may be based on different molecular determinants. For instance, it was shown for Cy-AMP1 that its chitin-binding capacity plays an essential role in antifungal, but not antimicrobial activity [27]. Interestingly, the substitution of a key serine residue in WAMP-1a renders this peptide markedly amphiphilic, suggesting membrane-active properties. We suppose that this peptide possesses both antibacterial and antifungal activity due to its cationicity and amphiphilicity and, hence, its ability to interact with membranes. This is compatible with morphological changes in fungi, induced by the peptide [6]. The same seems true for EAFP2, another hevein-type peptide possessing five disulfide bridges, its cationic residues were proposed to be critical for antimicrobial activity through binding to phospholipids in plasma membranes of pathogens, and amphiphilicity is thought to cause its insertion into the membranes, resulting in disturbance of the normal membrane functions and pore formation [7,22]. Hydrophobic/hydrophilic properties of peptides belonging to different structural families were recognized to be crucial for the mode of antimicrobial activity [28,29], and optimizing organization of cationic and hydrophobic regions can strongly improve activity [30].

The serine to glycine mutation attenuates chitin-binding capacity of WAMP-1a in exchange for a novel functionality. Similar examples in other protein families can be found. Indeed, for the snake toxins of the so-called three-finger fold, all of the amino acid residues were divided into two categories: structural and functional. It was supposed that only few of the 60–70 residues of these peptides are critical for the fold [31]. Two families of these toxins, neurotoxins and cytotoxins, possess different functionalities but the same backbone topology. A concept of moulds with multiple missions arose [32]. This means that variation of the functional residues results in generation of proteins with new functions but a conserved fold. Keeping this in mind we cannot exclude that WAMP-1a is a representative of a new family of peptides featuring the hevein fold but possessing a new yet unexplored function. This viewpoint is supported by the fact that the knottin-like topology is the most widespread structural motif found in the currently known disulfide-rich structures [21]. Other protein families converge to this structural motif because of its durability.

4.3. WAMP-1a structure suggests its evolutionary origin from chitinases

The hevein domain represents a curious example of molecular evolution. It is often a part of larger multi-domain proteins, such as chitinases and agglutinins [33]. Moreover, it may also be found per se, being characteristic of a number of plant defense peptides (Fig. 1). The conserved cysteines in hevein-type peptides are precisely positioned, and as a result, the coordinates of the residues constituting the chitin-binding domain are well defined as well. We superimposed the peptides listed in Fig. 1 over the conserved cysteines (C1–C6). The examples are illustrated in Fig. 3D and E, showing that the hevein-type fold, indeed, is determined by these cysteines.

It is logical to assume that the full disulfide bond networks determine evolutionary relationships within the family of the hevein-type peptides (Fig. 1). The S–S–bond arrangement in WAMP-1a exactly matches the disulfide connectivities in some plant chitinases. Thus, WAMP-1a may be considered as an isolated hevein domain of chitinases. The peptide may represent the result of a larger precursor maturation that contains the chitinase catalytic domain, as in case of Ee-CBPs [34]. It is also possible that the part of WAMP-1a gene encoding the catalytic domain is mutated so that the expression product is the sole hevein domain.

These observations fit the concept of protein promiscuity in the plant defense field [35]. According to it, defense peptides with common scaffolds acquire multiple activities during evolution. This is essential for plants to protect themselves from insect pests and pathogens. Apparently, WAMP-1a is a representative of hevein-type peptides, acquiring activity against certain types of bacteria, oomycetes, and fungi [6] due to a mutation in the chitin-binding domain, leaving the fold intact.

Database linking and Accession numbers

Coordinates, experimental restraints, and chemical shifts for the ensemble of 20 WAMP-1a structures have been deposited with the Protein Data Bank (PDB accession code 2LB7, www.rcsb.org) and the Biological Magnetic Resonance Bank, BMRB (code 17547, www.bmrb.wisc.edu), respectively.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.058.

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