Biochemical and Biophysical Research Communications 411 (2011) 14–18

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Peter V. Dubovskii [a,*], Alexander A. Vassilevski [a], Anna A. Slavokhotova [b], Tatyana I. Odintsova [b], Eugene V. Grishin [a], Tsezi A. Egorov [a], Alexander S. Arseniev [a]

[a] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 16/10 Miklukho-Maklaya Str., 117997 Moscow, Russian Federation
[b] Vavilov Institute of General Genetics, Russian Academy of Sciences, 3 Gubkina Str., 119991 Moscow, Russian Federation

ARTICLE INFO

Article history:
Received 30 May 2011
Available online 14 June 2011

Keywords:
NMR spectroscopy
^{13}C,^{15}N-natural abundance
Spatial structure
Chitin-binding domain
Molecular evolution
Triticum kiharae

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.

* Corresponding author. Fax: +7 495 335 50 33.
E-mail address: peter@nmr.ru (P.V. Dubovskii).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.06.058

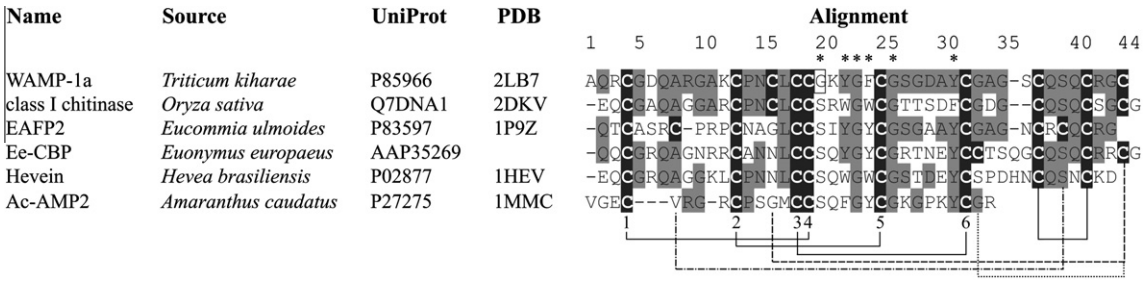

Fig. 1. Sequence alignment of WAMP-1a with selected hevein-type AMPs and chitin-binding domain of a class I chitinase from rice. Numbering is according to WAMP-1a. Cysteine residues are shaded in black; other residues identical to those in WAMP-1a are shaded in gray. Six conserved cysteines are numbered 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