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Heterologous expression and solution structure of defensin from lentil *Lens culinaris* ☆

Zakhar O. Shenkarev [a], Albina K. Gizatullina [a,b], Ekaterina I. Finkina [a], Ekaterina A. Alekseeva [a], Sergey V. Balandin [a], Konstantin S. Mineev [a], Alexander S. Arseniev [a,b], Tatiana V. Ovchinnikova [a,b,*]

[a] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya Str., 16/10, 117997 Moscow, Russia*
[b] *Moscow Institute of Physics and Technology (State University), Department of Physicochemical Biology and Biotechnology, Institutskii per., 9, 141700 Dolgoprudny, Moscow Region, Russia*

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ABSTRACT

A new defensin Lc-def, isolated from germinated seeds of the lentil *Lens culinaris*, has molecular mass 5440.4 Da and consists of 47 amino acid residues. Lc-def and its ^{15}N-labeled analog were overexpressed in *Escherichia coli*. Antimicrobial activity of the recombinant protein was examined, and its spatial structure, dynamics, and interaction with lipid vesicles were studied by NMR spectroscopy. It was shown that Lc-def is active against fungi, but does not inhibit the growth of Gram-positive and Gram-negative bacteria. The peptide is monomeric in aqueous solution and contains one α-helix and triple-stranded β-sheet, which form cysteine-stabilized αβ motif (CSαβ) previously found in other plant defensins. The sterically neighboring loop1 and loop3 protrude from the defensin core and demonstrate significant mobility on the μs–ms timescale. Lc-def does not bind to the zwitterionic lipid (POPC) vesicles but interacts with the partially anionic (POPC/DOPG, 7:3) membranes under low-salt conditions. The Lc-def antifungal activity might be mediated through electrostatic interaction with anionic lipid components of fungal membranes.

1. Introduction

Plant defensins belong to a large family of pathogenesis-related proteins (PRP), which are involved in plant defense against pests and pathogens and accumulated in plant tissues under stress conditions [1]. The defensins are basic peptides possessing antimicrobial activity toward different pathogens including bacteria, fungi, and viruses [2]. These peptides consist of 45–54 amino acid residues and characterized by the presence of some conservative amino acid residues including eight cysteines [3]. Spatial structure of plant defensins is typically formed by antiparallel triple-stranded β-sheet packed against an α-helix (in a $\beta_1\alpha\beta_2\beta_3$ configuration) [4]. Plant defensins contain three conserved intramolecular disulfide bridges (C2–C5, C3–C6, C4–C7) forming so-called cysteine-stabilized αβ motif (CSαβ) [5]. This motif was found in many toxins isolated from arthropods including arachnids and insects [5]. An additional disulfide bond (C1–C8) brings together the N- and C-terminal regions of plant defensins forming pseudo-cyclic structures.

Plant defensins possess various biological activities including inhibitory effects on a broad range of plant and human pathogens (e.g. different *Candida* species [6]), digestive enzymes (α-amylases [7] and serine proteinases [8]), HIV-1 reverse transcriptase [9], and growth of parasitic plants [10]. The peptides take part in regulating plant growth and development [11] and promote zinc tolerance in plants [12]. Some plant defensins inhibit protein translation in a cell-free system [13] and proliferation of tumor cells [14], block ion channels [15], display insecticidal effects [16] and mitogenic activity towards mouse splenocytes [17].

Molecular mechanism of antimicrobial action of plant defensins is still obscure. In contrast to other antimicrobial peptides, defensins do not permeabilize artificial phospholipid membranes [18], but induced hyphal permeabilization [19]. Recent research has demonstrated that microbe-specific lipid receptors are involved in antibiotic activity of various defensins [20]. In common with

Abbreviations: CSαβ, cysteine-stabilized αβ motif; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; SUV, small unilamellar vesicles.

☆ The atomic coordinates and structure factors have been deposited in the Worldwide Protein Data Bank (PDB: 2LJ7).

* Corresponding author at: Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya Str., 16/10, 117997 Moscow, Russia. Fax: +7 495 336 43 33.

E-mail addresses: ovch@ibch.ru, ovch@bk.ru (T.V. Ovchinnikova).

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human, invertebrate, and fungal defensins that bind to lipid II of the bacterial cell wall [20], plant and insect defensins were found to interact with sphingolipid receptors, resulting in fungal cell death [21]. Besides, plant defensins might enter the cell and access intracellular targets, such as DNA, RNA or components of the protein biosynthesis machinery [18].

In previous study [22], we discovered a novel defensin, termed as Lc-def, in the lentil *Lens culinaris* germinated seeds. Lc-def has molecular mass 5440.4 Da and consists of 47 amino acid residues including 8 cysteines forming 4 disulfide bonds. Here we report on the development of a bacterial expression system for production of the recombinant Lc-def and its ^{15}N-labeled analog. Antimicrobial activity and solution structure of the recombinant Lc-def were studied in the present work.

2. Materials and methods

2.1. Heterologous expression and purification of recombinant Lc-def and its ^{15}N-labeled analog

The recombinant plasmid pET-Trx-Lc-def was constructed by ligating the 5253 bp BglII/XhoI fragment of pET-31b(+) vector (Novagen) with a PCR-constructed insert encoding the recombinant protein (see Supplementary data). The BL-21 (DE3) cells transformed with pET-His8-TrxL-Lc-def were grown in LB medium containing ampicillin and glucose. ^{15}N-labeled Lc-def was expressed in M9 minimal medium containing $^{15}NH_4Cl$. Purification of the recombinant Lc-def and its ^{15}N-labeled analog involved immobilized-metal affinity chromatography (IMAC), refolding, CNBr cleavage of the fusion protein, elimination of the carrier protein by IMAC, and final RP-HPLC (see Supplementary data).

2.2. Antimicrobial assay

Antimicrobial activity of the recombinant Lc-def was measured by microspectrophotometry using 96-well microplates and serial dilutions of the peptide as described [23]. Spore germination and altered morphology of hyphae were observed with an Olympus CKX41 microscope (see Supplementary data).

2.3. NMR experiments and spatial structure calculation

NMR investigation was done using 0.5–1.0 mM samples of Lc-def or its ^{15}N-labeled analog in 10% D_2O or 100% D_2O at pH 5.0. All the NMR spectra were acquired on a Bruker Avance 600 spectrometer equipped with a cryoprobe at 27 °C or 55 °C. ^{1}H and ^{15}N resonance assignment was obtained by a standard procedure using combination of 2D and 3D TOCSY and NOESY spectra [24]. The data obtained from 2D DQF-COSY and ^{13}C-HSQC spectra were also used. The $^3J_{H^NH^\alpha}$ and $^3J_{NH^\beta}$ coupling constants were measured using 3D HNHA and HNHB experiments [24]. The $^3J_{H^\alpha H^\beta}$ coupling constants for AMX systems were estimated from splitting of $H^\alpha H^\beta$ cross-peaks in 2D TOCSY and NOESY spectra.

Spatial structure calculation was performed in the CYANA 2.1 program [25]. Upper interproton distance constraints were derived from the intensities of cross-peaks in 2D and 3D NOESY spectra (τ_m = 120 ms) via a "$1/r^6$" calibration. Torsion angle restraints and stereospecific assignments were obtained from *J* coupling constants and NOE intensities. Hydrogen bonds were introduced basing on water and deuterium exchange rates of H^N protons (Fig. 2B). The disulfide bond connectivity pattern was established on the basis of the observed NOE contacts and verified during preliminary stages of the spatial structure calculation.

^{15}N-relaxation parameters (R_1 and R_2 rates and heteronuclear ^{15}N-{^{1}H} NOEs) were measured at 27 °C using pseudo 3D experiments [24]. Hydrodynamic calculations were carried out in the HYDRONMR program [26].

Small unilamellar vesicles (SUV) composed of POPC or POPC/DOPG (7:3) mixture (Avanti Polar Lipids, Alabaster, AL) were prepared by sonication. Titration of the unlabeled Lc-def sample (30 μM, 5% D_2O, 10 mM Tris-Ac buffer, pH 7.0) with SUV was performed at 27 °C. At each lipid concentration 1D ^{1}H NMR spectrum was measured, and the equilibrium concentration of the "free" peptide in solution was determined on the basis of the integral intensity of the amide-aromatic region of the spectrum.

3. Results

3.1. Heterologous expression, purification and characterization of recombinant Lc-def and its stable isotope labeled analog

The recombinant Lc-def was produced in *Escherichia coli* using T7 promoter based expression system. *E. coli* BL-21(DE3) cells were transformed by pET-His8-TrxL-Lc-def containing the Lc-def sequence fused with the His8-tagged thioredoxin A (M37L) carrier protein (Fig. S1). Decreasing the induction temperature to 25–30 °C resulted in increasing level of the Lc-def-containing fusion protein expression in a soluble form. In LB medium the recombinant peptide was obtained mostly in a soluble form. The yield of the purified peptide constituted up to 3.5 mg/L of the medium. In order to obtain the uniformly ^{15}N-labeled recombinant Lc-def, the BL-21 (DE3) cells transformed with the same plasmid were grown in M9 minimal medium containing $^{15}NH_4Cl$ as the only source of nitrogen. The yield of the ^{15}N-labeled Lc-def was somewhat lower (2.5 mg/L). MALDI-TOF MS analysis of the recombinant Lc-def revealed a monomeric protein with molecular mass identical to that of the native defensin (5440.06 Da and 5440.4 Da, respectively). The ^{15}N-labeled Lc-def showed 73 Da increase in molecular mass (5513.69 Da) indicating that all the ^{14}N atoms were substituted with the stable isotope ^{15}N. The SDS–PAGE revealed the refolded fusion protein in a monomeric form both under reducing and non-reducing conditions. At the same time, the recombinant Lc-def demonstrated different electrophoretic mobility under reducing and non-reducing conditions. Under non-reducing conditions the main peptide band corresponded to the protein dimer (~11 kDa). Addition of an excess of 2-mercaptoethanol resulted in the appearance of a single band corresponding to the monomeric peptide (data not shown).

3.2. Biological activity of the recombinant Lc-def

Antimicrobial activity of the recombinant Lc-def against 11 phytopathogenic microorganisms was examined by the broth

Table 1
Antimicrobial activity of Lc-def.

Test-microorganisms[a]	IC_{50}, μM
Bacteria	
Agrobacterium tumefaciens, strain A281	NA
Clavibacter michiganensis, strain VKM Ac-1144	NA
Pseudomonas syringae, strain VKM B-1546	NA
Fungi	
Alternaria alternata, strain VKM F-3047	NA
Ascochyta pisi, strain VKM F-1173	NA
Aspergillus niger, strain VKM F-2259	18.5
Aspergillus versicolor, strain VKM F-1114	18.5
Botrytis cinerea, strain VKM F-3700	9.25
Fusarium culmorum, strain VKM F-844	18.5–37.0
Fusarium solani, strain VKM F-142	NA
Neurospora crassa, strain VKM F-184	9.25–18.5

NA, not active.
[a] VKM strains were from all-Russian collection of microorganisms.