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Characterization of bi-domain drosomycin-type antifungal peptides in nematodes: An example of convergent evolution

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ABSTRACT

Drosomycin-type antifungal peptides (DTAFPs) are natural effectors of the innate immune system, which are restrictedly distributed in plants and ecdysozoans. Mehamycin is a bi-domain DTAFP (abbreviated as bDTAFP) firstly found in the Northern root-knot nematode *Meloidogyne hapla*. Here, we report its structural and functional features and the evolution of bDTAFPs in nematodes. Different from classical DTAFPs, mehamycin contains an insertion, called single Disulfide Bridge-linked Domain (abbreviated as sDBD), located in a loop region of the drosomycin scaffold. Despite this, recombinant mehamycin likely adopts a similar fold to drosomycin, as revealed by the circular dichroism spectral analysis. Functionally, it showed some weak activity against three species of fungi but relatively stronger activity against seven species of Gram-positive bacteria, indicative of functional diversification between mehamycin and classical DTAFPs. By computational data mining of the nematode databases, we identified polymorphic genes encoding mehamycin and a new multigene family of bDTAFPs (named roremycins) from *Rotylenchulus reniformis*. A combination of data suggests that the origination of sDBDs from *M. hapla* and *R. reniformis* is a consequence of convergent evolution, in which some probably suffered positive selection during evolution. Our study may be valuable in understanding the role of these unique antimicrobial peptides in the innate immunity of nematodes.

1. Introduction

As effector molecules of the innate immune system of multicellular organisms, antimicrobial peptides (AMPs) play a vital role in eliminating infections caused by various pathogenic bacteria, fungi, viruses and protozoa via acting as pore-formers or metabolic inhibitors (Brogden, 2005; Bulet et al., 2004; Nguyen et al., 2011; Zasloff, 2002). Drosomycin is the first inducible antifungal peptide initially isolated from haemolymph of immune-challenged *Drosophila melanogaster*, with strict activity against filamentous fungi (Fehlbaum et al., 1994; Gao and Zhu, 2008). Drosomycin comprises 44 residues with a typical cysteine-stabilized α-helical and β-sheet (CSαβ) fold composed of an α-helix and a three-stranded antiparallel β-sheet (Landon et al., 1997). This peptide contains eight cysteines that form four disulfide bridges, rendering it a compact structure with high stability and resistance to heat and proteases (Zhang and Zhu, 2009).

Drosomycin-type antifungal peptides (DTAFPs) are widely distributed in nearly all species of plants (i.e. plant defensins) and a clade of moulting animals, Ecdysozoa, including Arthropoda, Nematoda and Tardigrada, but absent in fungi and protozoans (Carvalho Ade and Gomes, 2011; Zhu and Gao, 2014). Such a patchy distribution supports an event of the plant-to-ecdysozoan horizontal gene transfer (Zhu and Gao, 2014). Heliomicin, an insect antifungal defensin from lepidopteran *Heliothis virescens*, shares approximately 50% sequence similarity to drosomycin. It contains six cysteines engaged in three disulfide bridges and also adopts a CSαβ fold (Lamberty et al., 1999, 2001). Due to the sequence, structural and functional similarities, heliomicins are classified as a subfamily within DTAFPs (Zhu and Gao, 2014).

Nematodes, one of the most successful groups of animals, have adapted to a broad range of ecological niches from free living to symbiotic and parasitic lifestyles. Many nematode species confront microbe-rich environments during at least part of their life cycle, thus requiring the innate immune system to fight against potential pathogenic microorganisms. Zhu and Gao (2014) identified a minor multiple gene family of DTAFPs, comprising of 15 members (termed cremycin-1 to cremycin-15), in the fruit nematode *Caenorhabditis remanei.* Of the 15 genes, 10 were found to be transcriptionally active and six were upregulated after fungal challenge, implying a potential role in host defense against fungi. *In vitro* functional assays confirmed the strict fungicidal activity of cremycin-5 against filamentous fungi and several clinical

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isolates of *Candida albicans*. In addition to these typical DTAFPs, a unique DTAFP named mehamycin was recognized in the Northern root-knot nematode *Meloidogyne hapla,* which has an insertion of 18 amino acids located in a loop region of its drosomycin scaffold. This kind of DTAFPs are considered as bi-domain DTAFPs (bDTAFPs).

Here, we report for the first time the structural and functional characterization of a recombinant mehamycin, especially its antifungal and antibacterial activities and the structural feature of the insertion. For clarification purposes, we called this recombinant peptide with an amino-terminal Met extension rMehamycin. Data mining for nematode genomes led to the discovery of new bDTAFPs in *Rotylenchulus reniformis*, allowing us to investigate their evolutionary relationships in the context of sequences and structures.

2. Materials and methods

2.1. Recombinant expression vector construction

The gene encoding mature mehamycin was synthesized by BGI-Tech (Beijing, China) with three *Escherichia coli* rare codons optimized to improve its expression. The synthesized gene was inserted into pET-28a at *Nco* I and *Sal* I restriction sites to obtain a recombinant product with only one extra methionine at the N-terminus. RCR primers used in this study were shown in Table S3.

2.2. Protein expression and in vitro folding

The expression, *in vitro* folding and purification of recombinant mehamycin were performed according to the method previously described (Turkov et al., 1997; Zhu et al., 2013). In brief, pET-28a-mehamycin was transformed into *E. coli* BL21 (DE3) pLysS cells for protein expression. The induction of the rMehamycin expression was initiated by 0.5 mM IPTG at an OD_{600} of 0.2. *E. coli* cells were harvested after induction for 4 h at 37 °C by centrifugation and the pellets were re-suspended in resuspension buffer (0.1 M Tris-HCl, pH 8.5; 0.1 M NaCl). Sonication was used to lyse the cells. Recombinant protein accumulated as inclusion bodies was firstly washed with isolation solution containing 2 M urea and 2% Triton X-100, and then solubilized in denaturation buffer containing 6 M guanidine-HCl, 30 mM β-mercaptoethanol, 1 mM EDTA and 0.1 M Tris–HCl (pH 8.5) for 2 h. *In vitro* refolding was performed by 20-fold dilution in refolding buffer containing 0.2 M ammonium acetate (pH 9.0) for 48 h at room temperature. Refolded protein was dissolved in water after salting out by 80% saturation of ammonium sulfate. The recombinant protein was finally purified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The Agilent Zorbax 300SB-C18 (4.6 × 150 mm, 5 μm) was equilibrated with 0.05% TFA in water (v/v) and the recombinant protein was eluted from the column with a linear gradient from 0 to 60% acetonitrile in 0.05% TFA in water (v/v) within 40 min at a flow rate of 1 ml/min. The UV absorbance trace was followed at a wavelength of 225 nm. The single well-defined peak of recombinant protein was collected and lyophilized by Thermo Scientific SAVANT SPD1010 SpeedVac Concentrator (USA). The molecular mass was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) on Ultraflextreme MALDITOF/TOF Mass Spectrometer (Bruker, Germany). Mass spectra were obtained in the positive linear mode. CHCA (a-cyano-4-hydroxycinnamic acid) matrix was prepared by dissolving 5 mg in 1 mL of 50:50 acetonitrile/water containing 0.1% TFA.

2.3. Circular dichroism (CD) analysis

CD spectra of rMehamycin were recorded on Chirascan^{TM}-plus CD spectrometer (Applied Photophysics Ltd, United Kingdom) at room temperature from 180 to 260 nm with a quartz cell of 1.0 mm thickness. Spectra were measured at a peptide concentration of 0.1 mg/ml in water. Data were collected at 1 nm intervals with a scan rate of 60 nm/min.

2.4. Disulfide bridge determination

For liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, rMehamycin in 100 mM NH_4HCO_3 (pH 8.0) was digested by chymotrypsin (50:1) at 37 °C for 16 h. The digested product was desalted and separated by capillary high performance liquid chromatography (Column: Dionex C18 PePMap300, particle size 1.9 μm, pore size 120 Å, ID 150 μm, length 12 cm; mobile phase: Solvent A, water containing 0.1% formic acid; Solvent B, 80% acetonitrile and 20% water containing 0.1% formic acid; flow velocity: 0.6 μl/min; eluent: 0 min 5% B; 8 min 8% B; 24 min 13% B; 60 min 28% B; 79 min 40% B; 80 min 95% B; 85 min 95% B; 86 min 6% B; 90 min 6% B) and then analyzed by the Q Exactive mass spectrometer (QE-MS, Thermo Scientific). Spray voltage was 1.90 KV. Capillary temperature was set at 320 °C. Mass-to-charge ratio (m/z) of peptide fragments were detected by a scan range of 300–1400 m/z.

2.5. Antimicrobial assays

Lethal concentration (C_L) of a peptide was determined by inhibition zone assay performed according to the method previously reported (Ekengren and Hultmark, 1999; Hultmark, 1998). Briefly, filamentous fungi were incubated on potato dextrose agar (PDA) (20% potato, 2% glucose, 1.5% agar) plate at 30 °C for 1 week. Spores were harvested and suspended in sterile water with an OD_{595} of 0.5. Six ml of PDA containing 0.8% agar was mixed with 50 μl spores suspension and poured into Petri dishes of 9.0 cm diameter, giving an agar depth of 1 mm. Wells with a diameter of 2 mm were punched into the medium, filled with 2 μl of samples each well. In this assay, three different doses of peptides were applied to three independent wells in one fungal plate. After incubation at 30 °C overnight, hyphal inhibition was seen as clear and circular zones around the wells filled with peptides. Inhibition zones were measured and used to calculate C_L based on the Hultmark's method (Hultmark, 1998). Bacteria or *C. albicans* respectively grown in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2) or potato dextrose broth (PDB) (20% potato, 2% glucose) at an OD_{600} of 0.5 were used as described above. Temperatures used were 37 °C for bacteria or 30 °C for *C. albicans*. The microorganisms used in this assay were listed in Table S4.

2.6. Database search

To find new bDTAFPs, the amino-acid sequences of drosomycin and nematode DTAFPs including cremycins and mehamycin were used as queries to perform TBLASTN search of nematode genomic databases in GenBank (http://www.ncbi.nlm.nih.gov/) under default parameters. The databases include 93 species of Nematoda with one genome completed and others from whole genome shotgun. New hits were also taken as queries until no hits appeared. The program GeneRunner (http://www.generunner.net/) was employed to predict complete open reading frames from selected nucleotide sequences. All retrieved sequences were submitted to SignaIP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) to predict their signal peptides. We also undertook other databases search in GenBank, which include the EST database, the nucleotide collection (nr/nt) database and the reference genomic sequences (refseq_genomic) database.

2.7. Multiple sequence alignment and phylogenetic tree construction

Multiple sequence alignment of DTAFPs was carried out by Clustal X2 and further refined by hand with reference to the cysteine residue position. The full-length peptide sequences aligned by MUSCLE were employed to construct a phylogenetic neighbor-joining (NJ) tree by

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