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Solution structure of a short-chain insecticidal toxin LaIT1 from the venom of scorpion *Liocheles australasiae*

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ABSTRACT

The solution structure of an insecticidal toxin LaIT1, a 36-residue peptide with a unique amino-acid sequence and two disulfide bonds, isolated from the venom of the scorpion *Liocheles australasiae* was determined by heteronuclear NMR spectroscopy. Structural similarity search showed that LaIT1 exhibits an inhibitory cystine knot (ICK)-like fold, which usually contains three or more disulfide bonds. Mutational analysis has revealed that two Arg residues of LaIT1, Arg^{13} and Arg^{15}, play significant roles in insecticidal activity.

1. Introduction

Most toxins isolated from animal venoms are small peptides that bind to ion channels, such as sodium, potassium, calcium and chloride channels, with high affinity and specificity [1–5]. These toxins block or modulate gating mechanisms of ion channels to immobilize prey or predators. Scorpion venom contains a variety of peptides that are toxic to mammals, insects and crustaceans [6]. To date, more than 600 peptides have been identified from scorpion venoms. Scorpion toxins can be divided into two groups, a 6–8 kDa long-chain peptide group and a 3–5 kDa short-chain peptide group, based on the molecular size [7,8], and also classified into two groups, a cystine-stabilized α-helix/β-sheet (CSα/β) group and an inhibitor cystine knot (ICK) group, based on the backbone fold [9,10]. The CSα/β fold consists of a short α-helix linked to a double- or triple-stranded antiparallel β-sheet stabilized by three or four disulfide bonds. This fold is observed in many scorpion toxins acting on potassium and sodium channels [11,12]. The ICK fold, also referred to as the knottin fold, consists of a double- or triple-stranded antiparallel β-sheet stabilized by three disulfide bonds, and forms a β-hairpin and a cystine knot [13,14]. In contrast to the CSα/β fold, the ICK fold is found in only a few scorpion toxins [10], although the ICK-fold peptides have been isolated from many species such as plants, marine cone snails and spiders.

LaIT1 is an insecticidal short-chain toxin isolated from the venom of the scorpion *Liocheles australasiae* [15]. This toxin consists of 36 amino-acid residues and two intramolecular disulfide bonds with a molecular mass of 4200 Da, and shares a sequence similarity to only one peptide OcyC10 (64% identical) isolated from the Brazilian scorpion venom [16]. Unlike other scorpion insecticidal toxins, LaIT1 and OcyC10 contain only two disulfide bonds. The unique amino-acid sequence and the lack of tertiary structural information prompted us to explore the three-dimensional structure of LaIT1 as well as to further characterize its insect toxicity.

In this study, we have analyzed the solution structure by heteronuclear NMR spectroscopy using ^{15}N-labeled and ^{13}C, ^{15}N-labeled LaIT1 that are produced in an *Escherichia coli* expression system. The NMR analysis has revealed that LaIT1 adopts an ICK-like fold although it contains only two disulfide bonds. We have also examined the species specificity in insect toxicity of LaIT1 and identified its amino-acid residues important for the insecticidal activity. We discuss the structure and function of LaIT1 by comparing the tertiary structures and the target ion channels with structurally similar scorpion and spider toxins.

2. Materials and methods

2.1. Expression and purification of ^{15}N- and ^{13}C, ^{15}N-labeled LaIT1

The double stranded DNA encoding LaIT1, ATGGATTTTCCGCT GAGCAAAGAATATGAAACCTGCGTGCGCCCGCGCAAATGTCAGCCGC CGCTGAAATGCAACAAAGCGCAGATTTGTGTGGACCCGAAAAAGGGC

Abbreviations: ICK, inhibitory cystine knot; CSα/β, cystine-stabilized α-helix/β-sheet.

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TGGtaataa, where taataa is the tandem stop codons, was synthesized by mixing the following four oligo nucleotides followed by thermal cycling: LaIT1-S1, cgcgcgcgCCATGGGATTTTCCGCTGAGCA AAGAATATGAAACCT; LaIT1-A2, GCGGCGGCTGACATTTGCGCGGGC GCACGCAGGTTTCATATTCTT; LaIT1-S3, AATGTCAGCCGCCGCTGAA ATGCAACAAAGCGCAGATTTGTGTGG; and LaIT1-A4, cgcGAATTC ttattaCCAGCCCTTTTTCGGGTCCACACAAATCTGCG. The *Nco*I and *Eco*RI sites (underlined in the above oligonucleotides) were used for the cloning of the DNA encoding LaIT1 to the expression vector pET-32a (Novagen). Non-labeled, ^{15}N-labeled and ^{13}C, ^{15}N-labeled Trx-His$_6$-ek-LaIT1, where "Trx" and "ek" represents the thioredoxin-tag and the enterokinase recognition site, respectively, were expressed in *E. coli* BL21(DE3) (Novagen) grown in LB medium, M9 minimal medium containing ^{15}N-labeled ammonium chloride and in M9 minimal medium containing ^{13}C-labeled glucose and ^{15}N-labeled ammonium chloride, respectively, by adding a final concentration of 1 mM IPTG and cultivating at 37 °C for 3 h. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole), and disrupted by sonication. After centrifugation, Trx-His$_6$-ek-LaIT1 was purified with a Ni Sepharose 6 Fast Flow (GE Healthcare) column. The Trx-His$_6$-tag was removed by enterokinase (Invitrogen). LaIT1 was purified by cation exchange chromatography using a HiTrap SP column (GE Healthcare).

2.2. Determination of disulfide bond linkages in recombinant LaIT1

To analyze the disulfide pattern of recombinant LaIT1, non-labeled recombinant LaIT1 was first digested with endoproteinase Lys-C (Wako Pure Chemical Industries, Osaka) in 25 mM Tris–HCl, pH 7.1, without reduction or alkylation of disulfide bonds. Then, the molecular masses of fragments were analyzed using an LCMS-IT-TOF mass spectrometer (Shimadzu, Kyoto) equipped with an electrospray ion source.

2.3. NMR spectroscopy

The solvent of the purified ^{13}C, ^{15}N-labeled LaIT1 was changed to 20 mM MES-NaOH, pH 6.0, and 50 mM NaCl in 90% H_2O/10% D_2O (v/v) and the peptide was concentrated to a final concentration of 1.5 mM by ultrafiltration using a Vivaspin-20 (MWCO 3000, Sartorius Stedim Biotech). All NMR spectra were measured at 25 °C on a Unity Inova 600-MHz NMR spectroscopy (Varian) equipped with a triple-resonance probe. The following NMR data were acquired for ^{13}C, ^{15}N-labeled LaIT1: ^{1}H–^{15}N HSQC, HNCO, HN(CO)CA, CBCA(CO)NH, HNCACB, HBHA(CO)NH, HCCH-COSY, HCCH-TOCSY, ^{15}N-edited NOESY-HSQC (mixing time, 90 and 175 ms), and ^{13}C-edited NOESY-HSQC (mixing time, 90 and 175 ms). In addition, ^{1}H–^{15}N HSQC and ^{15}N{^{1}H}-NOE (^{1}H saturation time, 3 s) were acquired for ^{15}N-labeled LaIT1. All the NMR data were processed, visualized and analyzed with the programs NMRPipe [17], NMRDraw [17] and Sparky (http://www.cgl.ucsf.edu/home/sparky/), respectively.

2.4. Hydrogen bond restraints

Hydrogen bonds were detected by the H → D exchange experiment. ^{1}H–^{15}N HSQC spectrum of 1.0 mM ^{15}N-labeled LaIT1 dissolved in 20 mM MES-NaOH, pH 6.0, and 50 mM NaCl in 90% H_2O/10% D_2O (v/v) were acquired. Then, the solvent was changed to 20 mM MES-NaOH, pH 6.0, and 50 mM NaCl in D_2O by ultrafiltration using a Vivaspin-20 (MWCO 3000). ^{1}H–^{15}N HSQC spectra were acquired at 12, 24, and 36 h after the solvent exchange and the peak intensity of each residue in these ^{1}H–^{15}N HSQC spectra was analyzed to detect slowly exchanging amide protons. Then, their hydrogen-bonded oxygen atoms were deduced from the LaIT1 structures at intermediate and final stages in the structure calculation. Hydrogen-bond distance restraints were set as r_{HN-O} = 1.8–2.5 and r_{N-O} = 2.7–3.5 Å.

2.5. Structure calculation and structural similarity search

Inter-proton distance restraints were derived from peak intensities in the ^{15}N-edited NOESY-HSQC and ^{13}C-edited NOESY-HSQC spectra of ^{13}C, ^{15}N-labeled LaIT1 with a mixing time of 90 ms. The cross-peak intensities were translated into inter-proton distances based on the relationships, NOE $\propto$ (distance)$^{-6}$ and the standard distance between H_i^α and H_j^α in the parallel β-sheet of 2.3 Å. Hydrogen-bond distance restraints were also added to the structure calculation as described above. Structure calculation was performed using CYANA [18]. A total of 200 conformers were annealed in 10,000 steps of torsion angle dynamics calculations, of which 10 conformers with the lowest values in the target function were used to represent the solution structure of LaIT1. The conformer with the lowest target function was used as the representative structure of LaIT1. The tertiary structure was visualized with the programs Molmol [19] and PyMol (http://pymol.sourceforge.net/). The representative structure with the lowest target function was submitted to the Dali server [20] to search for proteins structurally similar to LaIT1.

2.6. Chemical synthesis of LaIT1 and its analogs

LaIT1 and its analogs were synthesized by the Fmoc-based solid-phase peptide synthesis method using Wang-PEG resin (Watanabe Chemical Industries, Hiroshima). The C-terminal amino acid was attached to the resin by the symmetrical anhydride method catalyzed by 4-dimethylaminopyridine (DMAP). Symmetrical anhydrides of the Fmoc-protected amino acids were prepared in a reaction with 0.5 eq. of a diisopropylcarbodiimide in anhydrous dichloromethane. The symmetrical anhydrides (5 eq.) and DMAP (0.1 eq.) in anhydrous *N*,*N*-dimethylformamide (DMF) were added to the resin and incubated for 1 h at room temperature. After the resin was washed with DMF, the Fmoc group of the amino acid attached to the resin was deprotected with 20% piperidine in DMF. Fmoc-protected amino acids (3 eq.), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU, 3 eq.), and 1-hydroxybenzotriazole (3 eq.) in DMF were added to the resin. After *N*,*N*-diisopropylethylamine (6 eq.) was added to the resins, the reaction mixture was incubated for 1.5 h at room temperature. This deprotection/coupling cycle was repeated until the coupling of all residues was finished. Removal of side-chain protecting groups and cleavage of the peptide from the resin was carried out with trifluoroacetic acid (TFA)/triisopropylsilane/water/ethandithiol [94/2/2.5/2.5(v/v)] for 2 h at room temperature. After the resin was filtered off, cold diethyl ether was added to the filtrate to precipitate the cleaved peptides. The precipitated peptides were washed with cold diethyl ether twice and dried *in vacuo*. The crude linear peptides were then oxidized to form disulfide linkages in the glutathione redox buffer (1 mM reduced glutathione and 0.1 mM oxidized glutathione in 0.2 M Tris–HCl, pH 8.0). After incubation for 24–48 h at room temperature, the solution was concentrated. The folded peptides were purified by RP-HPLC using a linear gradient of CH_3CN in H_2O containing 0.1% TFA, and lyophilized. LC/MS measurements were carried out on an LCMS-IT-TOF mass spectrometer equipped with an electrospray ion source to confirm the molecular mass of the desired products.

2.7. Insect toxicity test

Insect toxicity was tested using crickets (*Acheta domestica*, ~50 mg body weight), cockroaches (*Periplaneta americana*,