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Identification of a novel class of conotoxins defined as V-conotoxins with a unique cysteine pattern and signal peptide sequence

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ABSTRACT

Cone snails are predatory gastropod mollusks distributed in all tropical marine habitats with a highly sophisticated defense strategy using small peptides in their venoms. Here, we report the discovery and initial characterization of the V-superfamily conotoxins. A novel conotoxin vi15a was purified from the venom of a worm-hunting species *Conus virgo*. The sequence of vi15a was determined to have a unique arrangement of cysteine residues (C-C-CC-C-C-C-C), which defines the new V-superfamily conotoxins. The cDNA of vi15a was cloned with RACE method. Its unique signal peptide sequence led to the cloning of another V-superfamily conotoxin, Vt15.1, from *Conus vitulinus*. These results, as well as the existence of Lt15.1 from *Conus litteratus* and ca15a from *Conus caracteristicus* with the same cysteine pattern, suggest that V-superfamily might be a large and diverse group of peptides widely distributed in different *Conus* species. Like other eight Cys-containing toxins, V-superfamily conotoxins might also adopt an ''ICK+1'' disulfide bond connectivity. The identification of this novel class of conotoxins will certainly improve our understanding of the structure diversity of disulfide rich toxins.

1. Introduction

Cone snails (superfamily Conoidea, family Conidae, genus *Conus*) are marine carnivores that use potent venoms to capture prey, defend against predators, and compete with other hunter species [14]. They show a high degree of specificity for certain prey type-fish (piscivorous), other molluscs (molluscivorous), or worms (vermivorous) [21]. The main component of *Conus* venoms is a complex mixture of pharmacologically active and conformationally constrained peptides, generally between 7 and 40 amino acids in length, known as ''conopeptides'' with zero or one disulfide bridge, or as ''conotoxins'' with two to five disulfides. Through adaptive evolution, the predatory cone snails have generated more than 70,000 different venom peptides [23,26,32] that target specific voltage-gated ion channels (Na^+, K^+, Ca^{2+}), ligand-gated ion channels (nAChR, 5-HT_3R, NMDAR), G protein-coupled receptors (neurotensin, vasopressin) or neurotransmitter transporters (NET) [27,32]. Since these toxins bind their targets with very high affinity and specificity, some of them are used as molecular probes to study specific subtypes of ion channels and receptors [2,20], and some are promising drug leads to treat several conditions [10,18,34], with one of them having been approved to relieve chronic pain [36].

The mature *Conus* peptides are generated by proteolytic cleavage and post-translational modifications from precursors that usually comprise a signal peptide, a ''pro'' region and a toxin-encoding region. Based on the sequences of the signal

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peptides, these highly diversified conotoxins are classified into several superfamilies (A, C, D, I, J, L, M, O, P, S, and T, for the ''conotoxins'') [32]. Members in a given superfamily share a highly conserved signal sequence in the precursors and a unique arrangement of cysteine residues with a characteristic number and pattern in their mature toxins [23,26,32]. Each superfamily can be further categorized into different families on the basis of their specific pharmacological targets [14,19,32]. For example, O-superfamily consists of five pharmacologically distinct members, namely, ω, δ, κ, μO and γ conotoxins. Each of them has a totally different molecular target and acting mechanism. The ω, κ, μO and γ conotoxins are specific blockers of Ca^{2+}, K^+, Na^+ and pacemaker channels, respectively, and δ conotoxins can delay the inactivation of voltage-gated sodium channels (VGSCs) [32].

Conotoxins containing eight cysteines are known to have been relatively less explored, among which the most extensively studied are the I-superfamily conotoxins. These *Conus* peptides, defined by framework 11 or XI [25], have four disulfide bonds with eight cysteine residues arranged in two pairs of adjacent Cys flanked by nonadjacent Cys residues (C-C-CC-CC-C-C) [7,8,13]. A preliminary analysis of *Conus* cDNA clones revealed that framework 11 peptides are found broadly across the genus, and exhibit an extreme diversity of sequences [8,15]. Recently, these varied toxins, although sharing the same cysteine arrangement, have been subdivided into two distinct genetic groups, the I_1- and I_2-superfamilies, based largely on the signal peptide sequence and the presence (I_1) or absence (I_2) of the canonical pro-peptide region in the precursor [8]. The I_1-class conotoxins are defined by five excitatory peptides isolated from the fish-hunting species *Conus radiatus* [7,8,13]. The best characterized of these, r11a (also known as ι-RXIA), targets the $Na_V1.6$ subtype of VGSCs but has no effects on KCNQ2/3 and several members of the K_V1 family of K^+ channels [6]. Members of the I_2-class include toxins from worm-hunting species such as κ-BtX from *Conus betulinus* [9] and ViTx from *Conus virgo* [16], which target two specific subtypes of K^+ channels and affect them in opposite ways. From the structural point of view, the I-superfamily of conotoxins may also be divided into three distinct groups according to the pattern of Cys residues and the processing of the C-terminus [8]. Group A ($CX_6CX_5CCX_1CCX_4CX_{8-10}C$) and group B ($CX_6CX_5CCX_3CCX_4CX_6C$) belong to gene superfamily I_1, whereas gene superfamily I_2 comprises group C ($CX_6CX_5CCX_3CCX_{2-3}CX_3C$).

Here, we describe the identification of a novel conotoxin, vi15a, purified from the venom of a vermivorous species *C. virgo*. The primary structure of this toxin reveals eight cysteine residues positioned in a novel arrangement which is the defining characteristic of the V-conotoxins. We further revisited eight cysteine containing toxins and their common structural feature is discussed.

2. Materials and methods

2.1. Materials

A Superdex™ Peptide HR 10/30 column was purchased from GE Healthcare, trifluoroacetic acid (TFA) and acetonitrile (ACN) for HPLC from Merck. The TRIzol reagent and 3′-RACE kit were purchased from Invitrogen, SMART™ cDNA library construction kit from Clontech, *Taq* DNA polymerase from TakaRa, PCR Product Purification Kit from QIAgen, and pGEM-T Easy vector system from Promega, respectively. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and all other reagents were of analytical grade.

2.2. Cone snail specimens and venom extraction

The living specimens of *C. virgo* were collected from the South China Sea near Hainan, China. The venom ducts from seven specimens of *C. virgo* were quickly dissected on ice and cut into segments. The venom was then extracted with 1.1% (v/v) acetic acid for 30 min at 4 °C. The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C and the supernatant was saved. The extraction was repeated three times and the supernatants were pooled and lyophilized.

2.3. Peptide purification

The lyophilized crude venom of *C. virgo* was dissolved in 10% ACN containing 0.1% TFA. After centrifugation at 12,000 rpm for 10 min at room temperature, the pellet was dissolved in 40% ACN, 0.1% TFA and subjected onto a pre-equilibrated Superdex™ Peptide HR 10/30 column at a flow rate of 0.4 ml/min. The collected fractions from each peak were pooled and lyophilized. Further purification of peptide-containing peaks of *C. virgo* was carried out on a ZORBAX 300SB-C18 semi-preparative column (9.4 × 250 mm, Agilent Technologies) at a flow rate of 2.0 ml/min. Buffer A was 0.1% TFA and buffer B was 0.1% TFA in ACN. All the chromatographic fractions were monitored at 214 and 280 nm.

2.4. Mass spectrometry

The mass spectrometry analyses of the toxins were performed on a Q-trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) in the scan type of Enhanced MS. The apparatus was equipped with a TurboIonSpray source and operated in the positive ionization mode.

2.5. Amino acid sequence determination

The purified peptide was dissolved in 400 μl of 200 mM Tris–HCl, and 1 mM EDTA, pH 8.0. One hundred-fold excess of dithiothreitol (DTT) was added and the sample was incubated at 42 °C for 40 min. The reduced peptide was repurified by reversed phase HPLC. The obtained linear peptide was dried and then redissolved in 300 μl of buffer A for alkylation with 3–5 mM of *N*-ethylmaleimide in darkness for 2 h at room temperature. The modified peptide was purified by HPLC and lyophilized. The alkylated derivative of vi15a was directly subjected to N-terminal amino acid sequence analysis on an ABI Model 491A Procise Protein Sequencing System (Applied Biosystems, Foster City, CA, USA).

2.6. Preparation of total RNAs and cDNA cloning

Venom duct total RNAs were extracted by using the TRIzol reagent according to the manufacturer's protocol. cDNAs were