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New conotoxins define the novel I_3-superfamily

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ABSTRACT

We purified two novel conotoxins, designated as ca11a and ca11b, from the venom of *Conus caracteristicus*. Based on the amino acid sequence of mature ca11a, we cloned its full-length cDNA. Based on the signal peptide of ca11a, several ca11a-like conotoxins were cloned from *C. caracteristicus* and *C. pulicarius*. These novel conotoxins have an I-superfamily cysteine pattern but with a novel signal peptide sequence, suggesting they belong to a new branch of I-superfamily, designated as I_3-superfamily. Additionally, two O-superfamily conotoxins were also cloned based on the signal peptide of ca11a, suggesting a possible evolutionary relationship between O- and I-superfamilies.

1. Introduction

Cone snails are predatory gastropods living in tropic sea water all over the world. Their venom ducts secrete a group of small disulfide-rich peptides, namely conotoxins, to capture preys or defend from predators. Conotoxins specifically target various ion channels and neurotransmitter receptors, therefore they have a remarkable diversity of pharmacological function and utility [7,20]. Based on their conserved signal peptide sequences, conotoxins can be classified into several gene superfamilies, including A-, T-, O-, M-, P-, I-, S-, J-, and L-superfamily [8,17]. Recently, several novel conotoxins with unique cysteine pattern and distinctive signal peptides were also reported [4,16,22].

In general, conotoxins in one gene superfamily share a conserved signal peptide, but may have different cysteine patterns. For example, the A-superfamily conotoxins share a conserved signal peptide, but have two distinct cysteine patterns, –CC–C–C– for α-conotoxins and –CC–C–C–C–C– for αA- and κA-conotoxins [19]. Our previous work also showed that α- and κA-conotoxins share a conserved signal peptide and gene structure [21]. In contrast, the I-superfamily conotoxins have two distinct signal peptides although they have a conserved cysteine pattern, –C–C–CC–CC–C–C–. Based on their distinct signal peptides, I-superfamily can be divided into two branches, I_1-superfamily and I_2-superfamily.

Although a lot of I-superfamily conotoxins have been identified by cDNA cloning and/or peptide purification [1,3,5,9,10,11], only three of them have known targets. The I_1-superfamily conotoxin RXIA targets the Nav1.6 subtype of voltage-gated sodium channels [2,6]. Two I_2-superfamily conotoxins, BtX and ViTX, are specific for the Ca^{2+}-regulated voltage-sensitive BK channels [5] and the Shaker subfamily K^+ channels (Kv1.1 and Kv1.3) [11], respectively.

Here we reported peptide purification and cDNA cloning of a group of novel conotoxins from *Conus caracteristicus* and *C. pulicarius*. They have an I-superfamily cysteine pattern but with a distinct signal peptide, suggesting they represent a new branch of I-superfamily, designated as I_3-superfamily. Furthermore, we also cloned two O-superfamily conotoxins based on the signal peptide of I_3-superfamily, suggesting a possible evolutionary relationship between O- and I-superfamilies.

2. Experimental procedures

2.1. Venom extraction and conotoxins purification

Specimens of *C. caracteristicus*, *C. pulicarius*, and *C. litteratus* were collected from the South China Sea near Sanya City. The crude venom extract from 10 venom ducts of *C. caracteristicus* was prepared as previously described [5]. The lyophilized crude extract was first subjected to a Superdex Peptide column (HR10/30, Pharmacia, Uppsala, Sweden). Subsequently, the conotoxin fraction eluted from the Superdex column was applied to a C18 reverse-phase column (ZORBAX 300SB-C18, 9.4 mm × 250 mm, Agilent Technologies, Santa Clara, CA, USA), and eluted by an acetonitrile gradient composed of solvent A and solvent B. Solvent

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A was 0.1% aqueous TFA, solvent B was acetonitrile containing 0.1% TFA. During elution, solvent B was linearly increased from 15% to 45% in 60 min at a flow rate of 2 ml/min. All major elution peaks were manually collected and lyophilized.

2.2. Reduction and modification of conotoxins

The purified conotoxins were dissolved in the reduction buffer (0.1 M Tris–HCl, 1 mM EDTA, 10 mM dithiothreitol, pH 8.0), respectively. The reduction reaction was carried out at 42 °C for 40 min. Then N-ethylmaleimide stock solution (dissolved in 0.1 M Tris–HCl, 1 mM EDTA, pH 8.0) was added to the final concentration of 50 mM to modify the free thiol moieties. The S-alkylation reaction was carried out at room temperature for 1 h. Subsequently, the modified conotoxins were subjected to a C18 reverse-phase column and eluted by an acetonitrile gradient as described in Section 2.1, respectively.

2.3. Mass spectrometry and N-terminal sequencing

The molecular masses of the native and S-alkylated conotoxins were measured by Q-trap mass spectrometry (Applied Biosystems, Foster city, CA, USA). The N-terminal amino acid sequences of the S-alkylated conotoxins were analyzed by automated Edman degradation (ABI Model 491A).

2.4. 3′-RACE

The total RNA was extracted from 2 venom ducts of *C. caracteristicus*, *C. pulicarius* and *C. litteratus*, respectively, using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcription was carried out using Superscript II Kit (Invitrogen, Carlsbad, CA, USA) that contains an oligo(dT)-containing adapter primer 5′-GGC CAC GCG TCG ACT AGT AC (dT)17-3′.

For 3′-RACE, a degenerate oligonucleotide primer P1 (5′-GCN TGG CCN TGY GGN GGN GT-3′, where Y = C or T, and N = G or A or T or C) was designed based on the partial mature peptide sequence (AWPCGGV) of ca11a. The reverse primer was a universal amplification primer (AUAP) 5′-GGC CAC GCG TCG ACT AGT AC-3′ that matches the complimentary strand of the adapter primer used for reverse transcription. The PCR amplification was carried out as previously described using a touch-down cycling protocol [21]. The amplified ~300 bp fragment was purified and ligated into a pGEM-T Easy vector (Promega) for DNA sequencing.

2.5. 5′-RACE

5′-RACE was performed based on the partial cDNA sequence determined by 3′-RACE using a 5′-RACE kit (Takara, Dalian, China). The gene-specific primer (P2) for reverse transcription was 5′-CAC AAG CGT GCC AGA ACT G-3′ corresponding to the cDNA sequence downstream of the stop codon at 23–42 bp. Then the product of reverse transcription was cyclized by T_4 RNA ligase at 16 °C for 18 h, and used as a template for gene specific PCR amplification. The amplification primers were 5′-TGT TGT TTC GGA ACG TCC AC-3′ (P3, forward) and 5′-ATC CAC AGC AAT CGT CAT GAC-3′ (P4, reverse), which were designed based on the known cDNA sequence of ca11a. The PCR amplification product was purified and ligated into a pGEM-T Easy vector (Promega) for DNA sequencing.

2.6. cDNA cloning

The reverse transcription was carried out as described above using total RNA from *C. caracteristicus*, *C. pulicarius*, or *C. litteratus* as template. Based on the signal peptide sequence of ca11a, a specific forward primer P5 (5′-ATG AAG CTG GTT TTG GCG ATC-3′) was designed, paired with the reverse primer AUAP to amplify other ca11a-like conotoxins. The PCR amplification was performed using a touch-down cycling protocol as described above. The PCR product was purified and ligated into a pGEM-T Easy vector (Promega) for DNA sequencing.

3. Results

3.1. Purification and sequencing of ca11a and ca11b

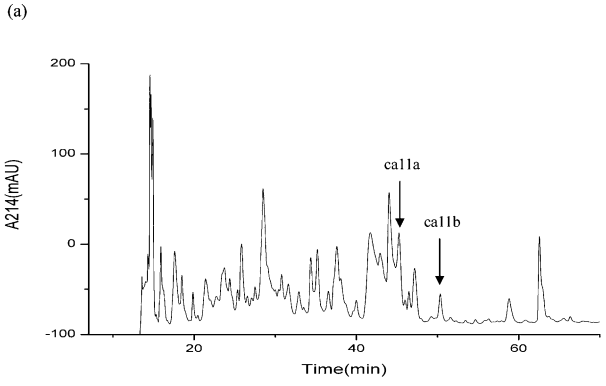
The crude venom extract from *C. caracteristicus* was first applied to a Superdex peptide column. Subsequently, the eluted conotoxin fraction was subjected to reverse-phase chromatography (Fig. 1a). All major elution peaks were manually collected, lyophilized, and sequenced. Among them, two I-superfamily conotoxins were identified. They are designated as ca11a and ca11b according to the nomenclature of conotoxins with unknown functions. As shown in Fig. 1b, ca11a has 38 residues and ca11b has 34 residues. No modified residues are found in their sequences.

3.2. Cloning the full-length cDNA of ca11a

A combined PCR and RACE approach was employed to clone the full-length cDNA of ca11a. Degenerate oligonucleotide primers were designed based on the amino acid sequence of the mature ca11a. Combining the results of 3′-RACE and 5′-RACE, a 471-bp cDNA was obtained (Fig. 2). The ca11a precursor includes a 20-residue signal peptide, a 22-residue pro-peptide, and a 38-residue mature peptide. The cDNA-deduced mature ca11a is consistent with the peptide sequencing result.

3.3. Cloning ca11a-like conotoxins

Based on the signal peptide sequence of ca11a, several ca11a-like conotoxins were cloned as shown in Fig. 3. Three conotoxins, ca11a, ca11b, and a ca11a-like conotoxin were cloned from *C. caracteristicus*. They were designated as Ca11.1, Ca11.2, and Ca11.3, respectively. Two ca11a-like conotoxins, Pu11.1 and Pu11.2, were cloned from *C. pulicarius*. These conotoxins have a typical I-superfamily cysteine pattern (–C–C–CC–CC–C–C–), but their signal



(b)

Conotoxin	Amino Acid sequence	Measured MW (Da)
ca11a	AWP**C**GGVRAS**C**SRHDD**CC**GSL**CC**FGTSTG**C**RVAVRP**C**W	4000.0 (4000.6)
ca11b	ALL**C**GGTHAR**C**NRDND**CC**GSL**CC**FGT**C**ISAFVP**C**	3504.3 (3504.0)

Fig. 1. (a) Purification of ca11a and ca11b by reverse-phase chromatography. The Superdex conotoxin fraction was applied to a C18 reverse-phase column, eluted by an acetonitrile gradient, and monitored by UV absorbance at 214 nm. The ca11a and ca11b peaks were indicated by arrows. (b) Chemically determined amino acid sequences and measured molecular masses of mature ca11a and ca11b. The theoretical molecular masses were shown in parentheses.