Published in final edited form as:

Peptides. 2010 September ; 31(9): 1678–1683. doi:10.1016/j.peptides.2010.05.020.

Divergent M- and O-superfamily peptides from venom of fish-hunting *Conus parius*

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Abstract

Six novel peptides from the piscivorous cone snail, *Conus parius* were purified by reverse-phase HPLC fractionation of crude venom. With the use of matrix-assisted laser desorption ionization mass spectrometry and standard Edman sequencing methods, the peptides were characterized. Two peptides were identified as members of the m-2 and m-4 branches of the M-superfamily and were designated as pr3a and pr3b, while four peptides were identified as members of the O-superfamily and were designated as pr6a, pr6b, pr6c and pr6d.

Peptide pr3a differs from the majority of the M-superfamily peptides in the presence of two prolines, which are not modified to 4-*trans*-hydroxyproline. In peptide pr3b, five amino acids out of the 16 non-cysteine residues are identical with those of μ -GIII and μ -PIIIA, suggesting that pr3b may be a divergent μ -conotoxin. Peptide pr6a is notable because of its extreme hydrophobicity. Peptide pr6c has three prolines that are unhydroxylated. Peptides pr6b and pr6d differ from the previously characterized O-superfamily peptides in the presence of an extended N-terminus consisting of six amino acids. Peptides pr3a, pr3b, pr6a and pr6b were demonstrated to be biologically active when injected intraperitoneally in fish. The identification and characterization of these peptides in venom of a fish-hunting species establish the divergence of gene products and their patterns of post-translational modification within superfamilies in a single *Conus* species.

Keywords

Conus parius peptide; M-superfamily conotoxin; O-superfamily conotoxin; conopeptide gene superfamily

1. Introduction

The venoms of some of the ~700 living species of predatory marine cone snails (*Conus*) have been systematically characterized. Each *Conus* species produces 100–200 small, disulfide-bridged peptides (conopeptides or conotoxins) in its venom. A rationale for the

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existence of such a huge and pharmacologically diverse array of peptides can be attributed to the complexity of biotic interactions that characterize the species-specific ecology of cone snails.

A basis for conopeptide diversity is the various gene superfamilies that encode the conopeptides [21]. The conopeptides within the same gene superfamily typically have a common disulfide-bridged scaffold, usually determined by a characteristic pattern of cysteine residues. Most conopeptides are disulfide-rich peptides, which fold in three-dimensional structures required for high affinity and specificity for their molecular targets, such as various subtypes of voltage-gated ion channels, ligand-gated ion channels and G protein-coupled receptors [20]. Although conopeptides in a superfamily are genetically and structurally related, the cone snails generate functional diversity by accelerated evolution leading to divergence in amino acid sequences, generally conserving the disulfide-bonding scaffold within each peptide superfamily.

Insights into the evolutionary strategies of cone snails have facilitated pharmacological discovery [11]. The present research on conopeptides uses a concerted discovery approach aimed at developing ligands for basic research and applied biomedical applications. In the last few decades, numerous conopeptide families have been characterized, showing that the diversity of their molecular targets matches the diversity of the conopeptides [20]. The unique specificity of conopeptides makes them desirable research reagents for defining the physiological roles of closely related subtypes of receptors and ion channels, and as therapeutic candidates [11,12].

In this work, we identified six novel peptides expressed in the venom of a single piscivorous *Conus* species, *Conus parius*. We describe the purification and characterization of these divergent peptides. One discovery strategy for identifying novel conopeptides in our laboratory has been to characterize high performance liquid chromatography (HPLC)-separated major fractions from crude venom extracts. Two of the peptides were identified as members of the m-2 and m-4 branches of the M-superfamily, while the other four were classified as members of the O-superfamily. As far as we know, this is the first report of an m-2 peptide from a fish-hunting *Conus* species, and the purification of both m-2 and m-4 peptides and four structurally distinct O-superfamily peptides from the venom of a single *Conus* species. The characterization of these novel peptides significantly expands the body of knowledge regarding the M- and O-superfamilies, and allows us to better predict the pattern of post-translational modification, as well as define important biochemical features to help identify specific molecular targets.

2. Materials and Methods

2.1. Preparation of venom extract

Specimens of *Conus parius* were collected from Philippine seawaters. They were brought alive to the laboratory, and their venom ducts were dissected as described previously [4]. The collected venom ducts were lyophilized and stored at – 80 °C. Fifteen lyophilized venom ducts were ground over liquid nitrogen using mortar and pestle. Venom was extracted sequentially with 5 ml of H₂O, 3 ml each of 20 % acetonitrile (ACN), 40 % ACN and 60 % ACN. The venom suspension in each extracting solvent was sonicated 5 times in 30-s intervals at 0 °C, and then centrifuged at 5000 x g for 5 min. The combined supernate (crude venom extract) was lyophilized and stored at – 20 °C until further purification.

2.2. Peptide purification

The crude venom extract was resuspended in a 1:9 mixture of 0.1 % trifluoroacetic acid (TFA) (solvent A) and 90 % ACN in 0.085 % TFA (solvent B) and applied into a Vydac

C18 analytical column. Peptides were eluted with a gradient of ACN using solvents A and B. The effluents were monitored at 220 nm. The selected major fractions containing the desired peptides were separately purified to homogeneity by analytical HPLC runs. The purified peptide solution was lyophilized and stored at -20°C .

2.3. Peptide sequencing

One nmol of the purified peptide (quantified by using known quantities of standard synthetic conotoxins, based on peak areas, with absorbance measured at 220 nm) was dissolved in 500 μL of a mixture of 90 % solvent A and 10 % solvent B. The pH was adjusted to 7.5 with 0.5 M Tris before dithiothreitol was added to a final concentration of 10 mM. The solution was flushed with argon, incubated for 15 min at 65°C and cooled to room temperature. Then 4-vinylpyridine (2 μL) was added and the solution was incubated in the dark for 20 min. The peptide solution was diluted with 500 μL and applied into an analytical HPLC column.

The purified alkylated peptide was sequenced by automated Edman degradation [6] on an Applied Biosystem model 492 sequenator, courtesy of Dr. Robert Schackmann of the DNA/Peptide Facility, University of Utah.

2.4. Mass spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectra were obtained by using a Voyager GE STR mass spectrometer at the Mass Spectrometry and Proteomic Core Facility of the University of Utah.

2.5. Bioassay

The purified peptides dissolved in normal saline solution (NSS) were separately injected intraperitoneally (i. p.) into gold fish (0.8 – 1.0 g), using a syringe with 29-gauge needle according to the method described earlier [2]. Controls were similarly injected with NSS. Three fish were injected with NSS or conopeptide. After injection, behavioral and/or physiological symptoms not observed in the control fish were noted.

3. Results

3.1. Purification, sequencing and mass determination of the peptides

A crude venom extract from *Conus parius* was fractionated using reverse-phase HPLC. In order to characterize the major peptides found in the venom, we selected several major fractions and purified the component peptides. The purified peptides were separately reduced, alkylated and sequenced by standard Edman chemical analysis. Their masses were determined by MALDI mass spectrometry.

3.1.1. Biochemical characterization of M-superfamily peptides pr3a and pr3b—

A fraction indicated by arrow (Figure 1A) was purified (Figure 1B–D). MALDI mass spectrometry of the purified peptide gave a monoisotopic mass of 1692.4. The peptide comprises 15 amino acids with the sequence CCNWPCSFGCIPCCY. The mass of the peptide obtained by MALDI mass spectrometry is consistent with the calculated monoisotopic mass of 1693.0. On the basis of the cysteine pattern the peptide was identified as a member of the M-superfamily; it was designated as pr3a.

A major hydrophilic fraction indicated by arrow (Figure 2A) was purified (Figure 2B–D). A monoisotopic mass of 2500.1 was obtained by MALDI mass spectrometry of the purified peptide. The peptide contains 22 amino acids with the sequence ERVCCGYOMSCKSRACKOSYCC*, where O is 4-*trans*-hydroxyproline and the asterisk indicates C-terminal amidation. The calculated monoisotopic mass of 2500.9 is consistent

with the MALDI mass. Based on the cysteine pattern the peptide was classified under the M-superfamily; it was named pr3b.

3.1.2. Biochemical characterization of O-superfamily peptides pr6a, pr6b, pr6c and pr6d

A very hydrophobic fraction indicated by arrow (Figure 3A) was purified (Figure 3B–C). MALDI mass spectrometry of the purified peptide showed a monoisotopic mass of 3090.4. The peptide has 29 amino acids with the sequence TCLARDELGASFLSNFLCCDGLCLLICV. The calculated monoisotopic mass of 3090.7 is consistent with the monoisotopic mass determined by MALDI mass spectrometry. The cysteine pattern of the peptide is consistent with the peptide being a member of the O-superfamily; the peptide was termed pr6a.

A fraction indicated by arrow (Figure 4A) was purified (Figure 4B–G). The purified peptide gave a monoisotopic mass of 3623.8 in MALDI mass spectrometry. The peptide comprises 32 amino acids with the sequence FGSFIOCAHKGEOCTICCROLRCHEEKTOTCV, where O is 4-*trans*-hydroxyproline. The calculated mass of 3622.2 is consistent with the MALDI monoisotopic mass. Based on the cysteine pattern the peptide belongs to the O-superfamily; it was designated as pr6b.

A major hydrophilic fraction indicated by arrow (Figure 5A) was divided into two portions; the 2nd half portion was purified (Figure 5B–D). Based on MALDI mass spectrometry, the purified peptide has a monoisotopic mass of 2614.0. The peptide contains 24 amino acids with the sequence DQCTYCGIYCCPPKFCTSSGCRSP. The calculated monoisotopic mass of 2614.0 is consistent with the mass determined by MALDI. The cysteine pattern indicates that the peptide belongs to the O-superfamily; the peptide was termed pr6c.

The 1st half portion of the major hydrophilic fraction indicated by arrow (Figure 6A) from which pr6c was obtained was further purified (Figure 6B–E). MALDI mass spectrometry of the purified peptide gave a monoisotopic mass of 3531.1. The peptide has 32 amino acids with the sequence YGNFOTCSETGEDCSAMHCCRSMTCTCRNNICAD, where O is 4-*trans*-hydroxyproline. The calculated monoisotopic mass of 3530.9 is consistent with the monoisotopic mass determined by MALDI. Based on the cysteine pattern the peptide was identified as a member of the O-superfamily; it was designated as pr6d.

3.2. Biological activities of the peptides

Bioassay of the peptides was carried out by i.p. injection of fish. The purified peptides pr3a, pr3b, pr6a and pr6b were obtained in sufficient quantities to assay after HPLC fractionation. Peptide pr3a (1 nmol) resulted in paralysis after ~ 5 min, while peptide pr3b (1 nmol) led to weakening and difficulty in swimming after ~ 20 min. Peptide pr6a (1 nmol) caused hyperactivity and erratic swimming in various directions after ~ 14 min, while peptide pr6b (0.5 nmol) resulted in vertical suspension and paralysis after ~ 6 min.

4. Discussion

We purified and characterized six novel peptides from the venom of *Conus parius*. Based on their cysteine patterns, the peptides were designated as pr3a, pr3b, pr6a, pr6b, pr6c and pr6d. Peptides pr3a and pr3b were identified as m-2 and m-4 peptides, respectively, of the M-superfamily [3], while peptides pr6a, pr6b, pr6c and pr6d were identified as members of the O-superfamily [8]. The superfamily classification of pr3a, pr3b, pr6c and pr6d was further confirmed based on the conserved frameworks in the sequences of their corresponding cDNA clones (M. Watkins and B. M. Olivera, unpublished data).

The M-superfamily conotoxins have the cysteine pattern, CC-C-C-CC, and include three distinct pharmacological families of peptides such as μ -conotoxins [5,18], κ M-conotoxins [1,7], and ψ -conotoxins [15] which block voltage-gated sodium channels, voltage-gated potassium channels, and nicotinic acetylcholine receptors, respectively. The M-superfamily peptides comprise a heterogeneous set of four distinct branches, m-1 to m-4; this classification is based on the number of amino acids separating the 4th and 5th cysteines [3].

The O-superfamily conotoxins have the cysteine pattern, C-C-CC-C-C, and include hydrophilic peptides such as ω -conotoxins [13,14] and κ -conotoxins [19] which block voltage-gated calcium channels and voltage-gated potassium channels, respectively, and very hydrophobic peptides such as δ -conotoxins [8,16,17] and μ O-conotoxins [10] which inhibit inactivation and conductance, respectively, of voltage-gated sodium channels.

The sequences of pr3a and pr3b show a cysteine pattern characteristic of the M-superfamily peptides identified earlier in other *Conus* species (Table 1). Based on the number of amino acid residues between the 4th and 5th cysteines, pr3a and pr3b belong to the m-2 and m-4 branches, respectively [3]. As far as we know, this is the first report of the presence of an m-2 peptide in a piscivorous species. Peptide pr3a has two proline residues which remain unhydroxylated (similar to tx3b which has three prolines). In contrast, a majority of the purified peptides in the M-superfamily have been found to contain prolines modified to 4-*trans*-hydroxyproline (Hyp). In pr3b, five of the 16 non-cysteine residues are identical with those of μ -GIIIA and μ -PIIIA (when their cysteines are aligned); these are Arg-2, Hyp-8, Arg-14, Lys-17, and Hyp-18, indicating that, likely, pr3b is a μ -conotoxin. The presence of both pr3a and pr3b in the venom of a fish-hunting *Conus* species is consistent with the finding that the peptides of the m-2 and m-4 branches are more genetically related to each other than to the peptides of the m-1 or m-3 branch [3].

Peptides pr6a, pr6b, pr6c and pr6d have the typical cysteine pattern of the O-superfamily (Table 2). Peptide pr6a is the most hydrophobic major peptide, while pr6c and pr6d are the most hydrophilic major peptides obtained in the fractionated venom. The hydrophobicity of pr6a is attributed to the presence of several bulky hydrophobic amino acids such as leucine, phenylalanine, isoleucine and valine. Both pr6a and pr6c are notable in the absence of any post-translational modification other than disulfide bond formation. In pr6c, three prolines remain unhydroxylated. Compared to the other members of the O-superfamily, pr6b and pr6d differ in the presence of an extended N-terminus consisting of six amino acids before the first cysteine. Probably, the extremely hydrophobic pr6a is a member of the δ -conotoxin family, while the hydrophilic pr6b, pr6c and pr6d are members of the ω -conotoxin or κ -conotoxin families.

The present results provide insight with respect to a general issue relevant to all *Conus* peptides: the pattern of post-translational modification. One of the more ubiquitous post-translational modifications is the hydroxylation of proline. It is a challenge to predict which proline residues will be hydroxylated. The purification of these six peptides from *Conus parius* provides relevant data.

The diversity of patterns of proline hydroxylation that have been observed (and at this point not explained) are typified by the 4 *Conus parius* peptides that belong to the O-superfamily. Thus, in two of these peptides, all prolines are hydroxylated (i.e., pr6b and pr6d). In pr6b, 4 prolines distributed across the entire mature sequence are hydroxylated. In contrast, none of the 3 prolines in pr6c are hydroxylated. The previously characterized O-superfamily peptides (Table 2), further demonstrate how unpredictable the pattern of hydroxylation can be; for example, all of the prolines in ω -conotoxin GVIA are hydroxylated, while in δ -

conotoxin PVIA, two of the prolines are hydroxylated, but one is not. In μ O-conotoxin MrVIA, none of the prolines are hydroxylated.

This confusing pattern for the O-superfamily contrasts with the results for the M-superfamily which can be rationalized. Thus in the m-4 branch of the superfamily, all prolines appear to be hydroxylated in all of the peptides isolated from venom so far. The novel peptide pr3b only reinforces this general rule. However, the m-2 peptide pr3a, in which none of the prolines are hydroxylated, provides some potentially useful data for predicting when hydroxylation will occur. In general, the last loop before the last two cysteine residues (e.g., ---CXPCC-) has prolines that are generally hydroxylated, but not in two of the peptides, pr3a and tx3b. Similarly, the prolines in the first loop are not hydroxylated in the examples shown. The isolation of pr3a suggests that if a smaller residue such as threonine or valine is present in the last loop, the proline is hydroxylated, but if it is a longer side chain (e.g., lysine or isoleucine) the proline is not hydroxylated. Prolines that are more N-terminal appear not to be hydroxylated. It will be interesting to see whether other peptides that are analyzed from venom which belong to the m-2 branch follow these rules.

Thus, the isolation of the two *Conus parius* peptides sheds some light into the pattern of hydroxylation of proline residues in M-superfamily peptides. Gaining such insights is important because it allows us to predict from peptide sequences obtained from clones, which prolines are hydroxylated in the native peptides, and which are not. In some cases, the correct hydroxylation of proline is essential to get the proper functional activity. It has been shown that substituting 4-*trans*-hydroxyproline for proline when the native peptide is not hydroxylated can cause a severe disruption in functional activity [9].

The discovery of pr3a and pr3b of the M-superfamily and pr6a, pr6b, pr6c and pr6d of the O-superfamily in venom from fish-hunting *Conus parius* establishes the remarkable structural diversity that exists in mature peptides within the same superfamily in a single venom. The emerging structural diversity of M- and O-superfamily peptides is likely to reveal more functional diversity among the members of these superfamilies. The biochemical characterization of more M- and O-superfamily peptides should reveal additional structural determinants that are essential for selectivity for their molecular targets. These new peptides contribute to the pool of peptides to be further explored for their pharmacological specificity.

Acknowledgments

This work was supported by NIGMS Program Project Grant GM48677.

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Figure 1.

Purification of peptide pr3a. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parius* with a gradient of 0–60 % solvent B (0.085 % TFA in 90 % acetonitrile) over 120 min. (B–D) Sequential elution of the fraction indicated by an arrow at 25–50 % solvent B over 25 min; 25–65 % solvent B over 20 min; 25–50 % solvent B over 25 min.

Figure 2.

Purification of peptide pr3b. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parius* with a gradient of 0–60 % solvent B (0.85 % TFA in 90 % acetonitrile) over 120 min. (B–D) Sequential elution of the fraction marked by an arrow at 15–40 % solvent B over 25 min; 15–75 % solvent B over 20 min; 15–55 % solvent B over 20 min.

Figure 3.

Purification of peptide pr6a. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parius* with a gradient of 0–60 % solvent B (0.85 % TFA in 90 % acetonitrile) over 120 min. (B–C) Sequential elution of the fraction indicated by an arrow at 45–75 % solvent B over 30 min; 45–75 % solvent B over 30 min.

Figure 4.

Purification of peptide pr6b. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parvus* with a gradient of 0–60 % solvent B (0.85 % TFA in 90 % acetonitrile) over 120 min. (B–G) Sequential elution of the fraction marked by an arrow at 20–50 % solvent B over 30 min; 20–80 % solvent B over 20 min; 20–45 % solvent B over 25 min; 20–70 % solvent B over 20 min; 20–90 % solvent B over 20 min; 20–45 % solvent B over 25 min.

Figure 5.

Purification of peptide pr6c. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parius* with a gradient of 0–60 % solvent B (0.85 % TFA in 90 % acetonitrile) over 120 min. (B–D) Sequential elution of the fraction indicated by an arrow at 10–40 % solvent B over 30 min; 10–60 % solvent B over 25 min; 10–60 % solvent B over 20 min.

Figure 6.

Purification of peptide pr6d. (A) Reverse-phase HPLC fractionation of crude venom extract from *Conus parius* with a gradient of 0–60 % solvent B (0.85 % TFA in 90 % acetonitrile) over 120 min. (B–E) Sequential elution of the fraction indicated by an arrow at 10–40 % solvent B over 30 min; 10–60 % B over 25 min; 10–40 % solvent B over 30 min; 10–60 % solvent B over 20 min.

Table 1

Comparison of sequences of M-superfamily conopeptides

Peptide	Sequence	Species	Reference
m-2 branch			
pr3a	CCNW <u>P</u> CSFGC <u>P</u> CCY	<i>C. parius</i>	This work
mr3a	GCCGSFACRFGCV $\overline{\text{O}}$ CCV	<i>C. marmoreus</i>	[3]
mr3b	SKQCCHLAACRFGCT $\overline{\text{O}}$ CCW	<i>C. marmoreus</i>	[3]
tx3b	CCPPVACNMGCK <u>P</u> CC*	<i>C. textile</i>	[3]
tx3c	CCRTCFGCT $\overline{\text{O}}$ CC*	<i>C. textile</i>	[3]
m-4 branch			
pr3b	ERVCCGY $\overline{\text{O}}$ MSCKSRACK $\overline{\text{O}}$ SYCC*	<i>C. parius</i>	This work
μ -GIIIA	RDCCT $\overline{\text{O}}$ $\overline{\text{O}}$ KKCKDRQCK $\overline{\text{O}}$ QRCCA*	<i>C. geographus</i>	[5]
μ -PIIIA	ZRLCCGF $\overline{\text{O}}$ KSCRSRQCK $\overline{\text{O}}$ HRCC*	<i>C. purpurascens</i>	[18]
κ M-RIIIK	L $\overline{\text{O}}$ SCCSLNLRLC $\overline{\text{O}}$ V $\overline{\text{O}}$ ACKRN $\overline{\text{O}}$ CCCT	<i>C. radiatus</i>	[7]
κ M-RIIIJ	L $\overline{\text{O}}$ $\overline{\text{O}}$ CCCT $\overline{\text{O}}$ $\overline{\text{O}}$ KKHC $\overline{\text{O}}$ A $\overline{\text{O}}$ ACKYK $\overline{\text{O}}$ CCCKS	<i>C. radiatus</i>	[1]
ψ -PIIIE	H $\overline{\text{O}}$ $\overline{\text{O}}$ CCLYGKCRRY $\overline{\text{O}}$ GCSASCCQR	<i>C. purpurascens</i>	[15]

O, 4-*trans*-hydroxyproline;

Z, pyroglutamate;

* amidated C-terminus.

The proline and 4-*trans*-hydroxyproline residues are underlined and “overlined”, respectively.

Table 2

Comparison of sequences of O-superfamily conopeptides

Peptide	Sequence	Species	Reference
pr6a	TCLARDEL ^u CGASFLSNFLCCDGLCLLICV	<i>C. parius</i>	This work
pr6b	FGSFI ^u ÖCAHKGEÖCTICCRÖLRCHEEKTÖTCV	<i>C. parius</i>	This work
pr6c	DQCTYCGIYCC ^u PKFCTSSGCRSP	<i>C. parius</i>	This work
pr6d	YGNFÖTCSETGEDCSAMHCCRSMT ^u CRNNICAD	<i>C. parius</i>	This work
ω-GVIA	CKSÖGSSCSÖTSYNCCRS ^u CNÖYTKRCY*	<i>C. geographus</i>	[14]
ω-MVIA	CKGKGAKCSRLMYDCCTGSCRS ^u GKC*	<i>C. magus</i>	[13]
κ-PVIA	CRIÖNQKCFQHLDDCCSRKCNRFNKC ^u V	<i>C. purpurascens</i>	[19]
δ-TxVIA	WCKQSGEMCNLLDQNC ^u CDGYCIVLVC	<i>C. textile</i>	[8]
δ-GmVIA	VKPCRKEGQLCD ^u PIFQNC ^u CRGWNCVLF ^u CV	<i>C. gloriamaris</i>	[17]
δ-PVIA	EACYAÖGTFCGIKÖGLCCSEFC ^u LPGVCF ^u G*	<i>C. purpurascens</i>	[16]
μO-MrVIA	ACRKKWEYCIV ^u PIIGFIYCC ^u PGLICG ^u PFVCV	<i>C. marmoreus</i>	[10]

O, 4-*trans*-hydroxyproline;*
amidated C-terminus.The proline and 4-*trans*-hydroxyproline residues are underlined and “overlined”, respectively.