Biologically Active Peptides from *Conus quercinus,* a Worm-Hunting Species

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ABSTRACT

The first biochemical characterization of biologically active compounds from the venom of a worm-hunting cone, Conus quericinus has revealed numerous components. Of these, three peptides were isolated: scratcher peptides QcIIIA and QcIIIB which are 15 amino acids long and homologous to each other, and scratcher peptide QcVIA which is entirely different and longer (25 amino acids). Posttranslational modifications found in the peptides include amidation of the carboxyl terminus and hydroxylation of proline. Although all three contain six cysteine residues, scratcher peptide QcIIIA and QcIIIB have the μ type arragement of Cys residues (CC. . .C. . .C. . .CC), and QcVIA has the ω framework (C. . .C. . .CC. . .C. . .C) typified by ω-conotoxins. The data obtained so far indicate that the biologically active components of all three major feeding types of Conus are mostly disulfide-rich peptides in approximately the same size range (10 to 30 amino acids). In all cases, the venoms are very complex.

INTRODUCTION

Studies of *Conus* venom have been done mostly on the geography cone, *Conus geographus*, a species which has been lethal to man (Cruz *et al.*, 1985; Olivera *et al.*, 1985; Gray,

Olivera and Cruz, 1988). More recently, we characterized venoms of other fish-hunting *Conus* species, such as *Conus striatus Conus tulipa* and Conus magus (Cruz *et al.,* 1987; Zafaralla *et al.*, 1988; Olivera *et al.*, 1990). This work revealed that the biologically active principles in piscivorous *Conus* venoms are small peptides which bind receptors and ion channels in the neuromuscular system. A prominent feature of most of these peptides is that they are very tightly disulfide bonded with an unusually high cysteine content.

The genus *Conus* is composed of approximately 500 species, and the fish-hunting *Conus* species are, in fact, a minority (approximately 10%). Although all *Conus* species are venomous, by far the greatest number of species are believed to be worm-hunting (Kohn, 1959). The majority of these probably feed on polychaete worms. It is also generally believed that the cone snails evolved from turrid ancestors approximately 60 million years ago (Olivera, Gray and Cruz, 1988). Since turrids are generally thought to be worm-hunters, the stem line of the genus *Conus* could very well have been the worm-hunting cones. Thus, it is of particular interest to determine whether the conclusions about the active principles in the fish-hunting *Conus* species are true for the worm-hunting *Conus* as well. It is conceivable that since the fish-hunting *Conus* species probably evolved later, the fish-hunting cones might differ significantly in their general biochemical strategy from the worm-hunting *Conus* species.

For this reason, we examined the venom of the worm-hunting species, *Conus quercinus*. It is likely that *Conus quercinus* feeds exclusively on annelid worms in nature. In an aquarium *Conus quercinus* will feed on a variety of marine polychaete worms; when such worms are present, *Conus quercinus* will promptly extend its proboscis and harpoon the worms.

In this article, we describe the first biological characterization of biologically active components from a worm-hunting *Conus* venom. It will be demonstrated below that although the biological activity of the components that have been isolated from *Conus quercinus* venom are different in specificity from the components characterized in *Conus geographus* venom, there are nevertheless striking biochemical similarities.

MATERIALS AND METHODS

Materials

Specimens of *Conus quercinus* were collected off the coast of Marinduque. The venom ducts were dissected from freshly

killed specimens, and the pooled venom was lyophilized and stored at -20°C. Mice used for bioassays were of Japanese sDDy strain. All other materials, reagents and solvents used were of reagent grade or HPLC grade.

Preparation of Venom Extracts

Approximately one gram of lyophilized *C. quercinus* venom was extracted repeatedly with 1.1% HOAc as previously described (McIntosh *et al.,* 1982) . All extracts were pooled, lyophilized, and stored in the freezer until further use.

Sephadex G-25 Chromatography

Freeze-dried extract was dissolved in 5 ml of 1.1% HOAc, applied to a pre-equilibrated Sephadex G-25 column (110 x 2.5 cm) and eluted with 1.1% HAc at 4°C. Ten-ml fractions were collected at a flow rate of 0.33 ml/min and absorbances were monitored at 276 nm using an LKB Uvicord. Fractions constituting a peak were pooled and aliquots were taken for bioassay and protein determination. The rest of the pooled fractions were lyophilized and stored in the freezer for further use.

High Performance Liquid Chromatography (HPLC)

Freezed fractions from the G-25 column were redissolved in 0.1% triflouroacetic acid (TFA) and filtered before application on an LKB Ultropac column (TSK ODS-120T; 10µm particle size; 7.8 x 300 mm). The peptides were eluted with a gradient of acetonitrile in 0.1% TFA. Fractions with interesting biological activities were fractionated by HPLC using a column with smaller pore size (TSK ODS-120T; 5µm) shallower gradients of acetonitrite in 0.1% TFA. Further purification of selected peaks was done using a VYDAC C18 column (250 x 4.6 mm, 5 um pore size, not end capped) eluted with an acetonitrile gradient in either 0.1% TFA or 0.05% heptafluorobutyric acid (HFBA).

Amino Acid Analysis

Peptide samples were hydrolyzed in vacuo in 6N HC1, 0.1% phenol for 18 hr at 105°C. Amino acid analysis was done by reverse-phase HPLC of phenylthiocarbamyl derivatives (Heinrikson and Meredith, 1984).

Peptide Sequencing

Peptides were reduced and carboxymethylated, and analyzed in a spinning-cup sequencer as described previously (Edman and Begg, 1967).

Bioassays

The biological activities were tested by intracerebral injection into mice as described by Clark, Olivera and Cruz (1981). LD_{50} of the crude venom was determined according to the method of Miller and Tainter (1944).

Protein Determination

The concentration of protein in the crude venom extract and fraction was determined according to the method of Lowry *et. al.* (1951).

RESULTS AND DISCUSSION

General Biological Effects

As with most worm-hunting cones, the venom apparatus of *C. quercinus* is relatively smaller than those of fish-hunting and mollusc-hunting cones. Its venom bulb is also particularly small relative to its shell size. The apparatus is relatively softer and more fragile compared to those of *C. magus* and *C. geographus,* both fish-hunters. On ic injection of the venom extract into mice, effects such as rapid breathing, scratching, jumping and running around, trembling and jerking of body and limbs, paralysis, and death were observed. The LD_{50} in mice was 3.55 mg protein per kg body weight. At very high dose (100mg) it can also be lethal on intraperitoneal injection.

Purification of Scratcher Peptides

The general strategy which has been used for purifying peptides from *Conus* venom involves a molecular sieve for initial separation of the peptides from proteases, followed by a series of HPLC runs on reverse phase C18 columns eluted with a gradient of acetonitrile either in 0.1% TFA or 0.05% HFBA. Fig. 1A shows the chromatogram of crude venom extract on a column of Sphadex G-25 eluted with 1.1% HOAc. Four major peaks with different biological activities were obtained. The main peak (stippled) produced micturation, violent jerking of limbs, body twisting, labored breathing, violent run-

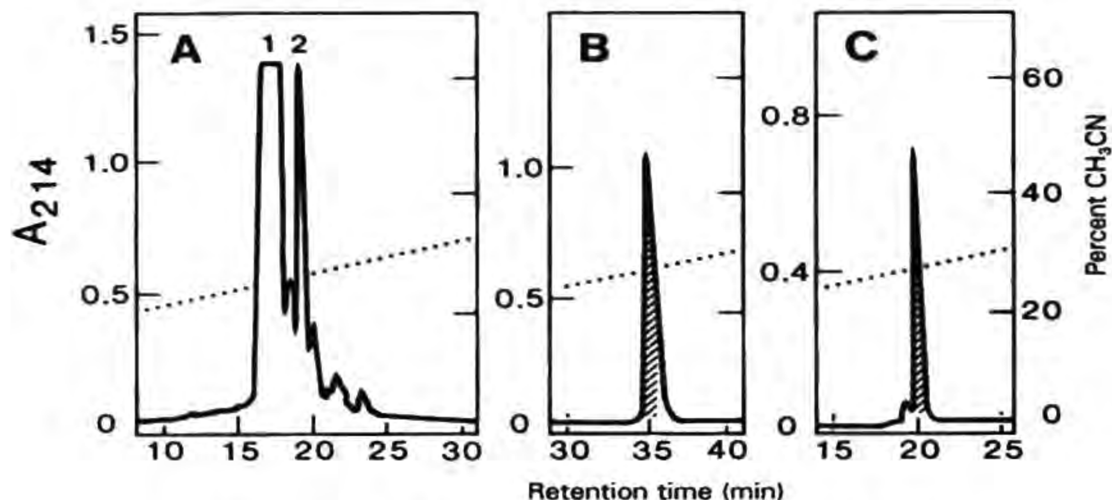

Fig. 3. Purification of scratcher peptides QcIIIA and QcVIA. (A) Peak of Fig. 1B was resolved into several peaks by HPLC on C18 column. (B) Peak 1 of Fig. 3A as purified on HPLC contained mainly scratcher peptide QcIIIA. (C) The major peak obtained on repurification of Peak 2 from Fig. 3A is scratcher peptide QcVIA. The columns were eluted with gradients of acetonitrilein 0.1% TFA as indicated by the broken lines.

Table 2. Sequence analysis of Scratchers 1 and 2.

STEP	SCRATCHER 1		SCRATCHER 2	
	Assigned Residue	Yield*	Assigned Residue	Yield*
1	Cys	1.11	Cys	1.86
2	Cys	0.94	Cys	1.46
3	Ser	0.92	Ser	0.91
4	Arg	0.80	Gln	1.35
5	His	0.43	Asp	0.92
6	Cys	0.73	Cys	0.73
7	Trp	0.64	Leu	0.71
8	Val	0.58	Val	0.92
9	Cys	0.49	Cys	0.54
10	Ile	0.48	Ile	0.92
11	Hyp	0.16	Hyp	0.35
12	Cys	0.41	Cys	0.26
13	Cys	0.43	Cys	0.45
14	Pro	0.06	Pro	.053
15	Asn	0.0045	Asn	.036

*Yield is expressed in nmole of PTH amino acid obtained. Values are not corrected for tailover.

However, the peptides described above were purified because they are active in the mammalian central nervous system. Thus, these peptides are likely to have very broad phylogenetic activity. Since *Conus quercinus* is a vermivorous species, presumably the true receptor targets of the three scratcher peptides in Table 3 are receptors or ion channels present in polychaete worms. The observation that the targets for these peptides are moderately conserved between polychaete worms and the mammalian central nervous system. The *C. quercinus* scratchers should be useful tools in a phylogenetic analysis of their receptor targets.

General Features of *Conus quercinus* Scratcher Peptides

The three peptides that have been purified from *Conus quercinus* are generally similar in biochemical features to previously characterized conotoxins from the fish-hunting cone snails (Olivera et al., 1985). They are relatively small peptides (15-25 amino acids) in the same size range as the paralytic peptides (i.e., the α-, μ- and ω-conotoxins) in piscivorous *Conus* venoms. In addition, a number of posttranslational modifications are seen in these peptides that were previously observed

Table 3. Sequences of scratcher peptides from *Conus quercinus* venom.

SCRATCHER PEPTIDE[a]	SEQUENCE
QcIIIA	Cys-Cys-Ser- Gln-Asp- Cys- Leu- Val-Cys-Ile- Hyp-Cys-Cys-Pro-Asn- NH_2
QcIIIB	Cys-Cys-Ser- Arg-His- Cys- Trp- Val-Cys-Ile- Hyp-Cys-Cys-Pro-Asn
QcVIA	Asp Gln-Ser - Cys-Hyp-Trp- Cys - Gly-Phe-Thr - Cys-Cys-Leu-Pro-Asn-Tyr-Cys-Gln-Gly-Leu-Thr-Cys-(Thr, Val, Ile)

[a]Nomenclature of peptides from Conus venom was discussed by Cruz et al. (1985) and Gray, Olivea and Cruz (1988). Qc indicates that the peptide is from *C. quercinus* venom. The Roman numeral III indicates that the peptide has the same Cys grouping as the μ conotoxins (CC...C...C...CC), and VI indicates the same Cys grouping as the ω-conotoxins (C...C...CC...C...C). The particular sequence variant is designated by the letters A and B.

in the paralytic conotoxins from *Conus geographus:* the presence of hydroxyproline in all three Scratcher peptides and the amidated C-terminus that was established in Scratcher peptide QcIIIA. In general, the *C. quercinus* peptides appear to be somewhat more cysteine rich than the peptides in *Conus geographus,* less postively charged and more hydrophobic. It should be noted that the Scratcher peptides are among the most hydrophobic components in the crude size fraction shown in Fig. 1; the total spectrum of peptides in *Conus quercinus* may not necessarily be more hydrophobic in character than the corresponding set of peptides in *Conus geographus* venom.

Conserved Frameworks in the *Conus quercinus* Peptides

The most surprising feature of the three sequences shown in Table 3 is that the arrangement of cysteine residues is relatively conserved if compared to the disulfide framework found in peptides from the fish-hunting cones. Thus, among the paralytic conotoxins containing six Cys residues, two general arrangements of cysteine residues were observed: the ω framework (typified by the ω-conotoxins) which have the following arrangement: C. . .C. . .CC. . .C. . .C, and the μ framework: CC. . .C. .C. . . CC (Woodward et al., 1990). The most striking similarity between the paralytic conotoxins from fish-hunting *Conus* and the three peptides from *Conus quercinus* are that Scratcher peptides QcIIIA and QcIIIB have the μ framework, and Scratcher peptide QcVIA and has the ω framework. We previously suggested in work with the King-Kong peptide from *Conus textile* that the toxins from different cone venoms may use only a few "Cysmotifs" as conserved structural backbones for targeting to a variety of receptors in different animals (Hillyard et al., 1989).

Recently, cloning data on conotoxins have suggested a possible mechanism for the generation of the bewildering array of peptides found in *Conus* venoms (Woodward et al., 1990). This hypothesized mechanism predicts that the Cys motifs will be generally conserved in all *Conus* venoms. The present results with *Conus quercinus* strongly support the prediction that the same conserved Cys motifs will be found in all Conus species.

Comparative Biochemistry of Conus Peptides

The results described in this report make it a virtual certainty that in all *Conus* venoms, small disulfide-rich peptides will be found. Previously, such peptides were described in a number of fish-hunting *Conus* species, and in one peptide from the venom of the

mollusc-hunting *Conus textile*. With the three peptides from the venom of *Conus quercinus* described here, all three major feeding types of *Conus* have now been examined, and in all cases disulfide-rich peptides in approximately the same size range (10-30 amino acids) are the biologically active components. This suggests that the biochemical strategy of using such small disulfide-rich peptides was already well established in the genus *Conus* before the three major feeding types diverged from each other. Although the taxonomy of *Conus* is somewhat confused, in all classification schemes, the fish-hunting *Conus geographus*, the snail-hunting *Conus textile* and the worm-hunting *Conus quercinus* represent rather unrelated branches of this large and diverse genus. The striking fact that in all three feeding types, not only are the peptides small and disulfide rich, but the arrangement of cysteine residues is conserved suggests that the basic strategy for evolving these peptides was possibly well established even in the turrid ancestors of the cone snails. This prediction needs to be experimentally tested and work on the biochemistry of turrid venoms is now in progress.

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