Curtatoxins

NEUROTOXIC INSECTICIDAL POLYPEPTIDES ISOLATED FROM THE FUNNEL-WEB SPIDER HOLOLENA CURTA*

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Three polypeptide neurotoxins (curtatoxins) were isolated from the venom of the spider Hololena curta by reverse-phase high performance liquid chromatography, gel permeation, and ion-exchange chromatography. The purified toxins induced an immediate paralysis in the cricket Acheta domestica that resulted in desiccation and death of the insect within 24-48 h (LD50 = 4-20 μg/g); this toxic effect is consistent with irreversible presynaptic neuromuscular blockade. Curtatoxins are a class of sequence-related, cysteine-rich, carboxyl-terminal amidated polypeptides of 36 to 38 amino acid residues. The cysteine residues are conserved at identical sequence positions among these polypeptides and form 4 intramolecular disulfide bonds. Hydrophathy calculations show that the toxins have an internal hydrophobic region flanked by hydrophilic and oppositely charged amino- and carboxyl-terminal ends. By analogy to other cysteine-rich arthropod venom proteins, the folded structure of the curtatoxins is likely important for their target specificity and mode of action at the neuromuscular junction.

In recent years the toxic components of spider venoms have attracted considerable attention for their use as probes of vertebrate and invertebrate nervous system functions (1, 2). Spider venoms paralyze insects by causing a block in neuromuscular transmission which is mediated by glutamatergic receptors (3). Presently, the structure of only a few of these toxins has been determined. Argiotoxin from the orb-weaving spider Argiope lobata was the first toxin structure to be elucidated (4) and since then other closely related toxins containing arginine and asparagine linked to polyamine chains have been reported (5–7). These toxins have been shown to cause a reversible block of the cation-selective, quisqualic acid-sensitive glutamate receptor of the locus (8). Venoms of other orb-weaving (Araneid) spiders contain low molecular weight postsynaptic toxins that also inhibit L-glutamate receptors and associated ion channels in the vertebrate central nervous system and at the invertebrate neuromuscular junction (9–18). In contrast to the reversible paralysis induced by the orb-weavers, venom of other spiders such as the Clubionid spider Chiracanthium inclusum and the funnel-web (Agelenid) spider, Hololena curta, induce a potent irreversible blockade by venom components that act both presynaptically and postsynaptically (17, 18). Presently, there is limited information on the structure and specificity of the neurotoxic components of Agelenid spiders. In search of potent inhibitors of neuronal functions, we have isolated and characterized three polypeptide isotoxins from the venom of H. curta that induce irreversible neuromuscular blockade in the cricket Acheta domestica.

EXPERIMENTAL PROCEDURES

The initial purification of the toxic components from whole venom of H. curta was performed by reverse-phase HPLC.2 Fig. 1 shows the areas of the irreversible paralytic activity in the chromatogram. The active principles (curtatoxins I–III) that caused irreversible paralysis were in fractions 27, 33, and 37. Anion-exchange chromatography of fraction 33 resulted in the elution of the paralytic activity in the unretained volume (Fig. 2). The toxic component of this fraction was further purified by gel permeation chromatography (Fig. 3). Final purification of this toxin was obtained by reverse-phase HPLC (Fig. 4) which yielded a single, symmetrical peak indicating a homogeneous component (curtatoxin II). The paralytic factor in fraction 37 was purified further by gel permeation chromatography (Fig. 5). Final purification of this toxin was obtained by reverse-phase HPLC (Fig. 6) which yielded a single peak indicative of a homogeneous component (curtatoxin III). The toxic component of fraction 27 (curtatoxin I) was purified by microbore reverse-phase HPLC (data not shown) and found to represent a pure component as described below.

Curtatoxin Biological Activity—When injected into the thoracic cavity of crickets (A. domestica), curtatoxins produced a rapid and irreversible flaccid paralysis. For example, with 3.0 μg of curtatoxin II or III the cricket usually desiccated and died after 24 h, whereas 1.0 μg (3.2 μg/g) resulted in paralysis of the insect after 30 min and death by 48 h. The LD50 for curtatoxin I was ~20 μg/g, whereas the LD50 for curtatoxins II and III were ~4 μg/g. The LD50 of the whole venom was 0.5 μl/g (24.9 μg/g protein/g).

Covalent Structure of Curtatoxins—The possibility that the

2 Portions of this paper (including "Experimental Procedures," Tables I–III, and Figs. 1–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

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irreversible paralytic toxins were hybrid-toxins consisting of peptides with arginine or asparagine linked to polyamine chains was investigated by LC-MS (22). However, no such components were found. In addition, no unusual amino acids were found by microchemical sequence analysis or byophthalaldehyde/9-fluorenylmethyloxazolone (23) amino acid analysis. The quantitative Edman degradations on the pyridylethylated spider toxins are given in Table I. Unambiguous assignments were obtained for the first 35 residues of curtatoxin I. Further confirmation of its structure was obtained by sequence analysis of overlapping peptides from tryptic digests (data not shown). Cycle 36 of curtatoxin I indicated Asn as the carboxyl-terminal residue. This assignment was verified by FAB-MS analysis on the isolated carboxyl-terminal tryptic tripeptide Asn-Asn-Asn-NH2 (see below), thus completing the sequence. As shown in Table III, FAB-MS analysis confirmed that the carboxyl terminus of curtatoxin I was amidated. The observed protonated molecular weight for the tripeptide (360.2) was identical to the theoretical value. The average protonated molecular weight determined for curtatoxin I was 4103.0 consistent with the theoretical value for the carboxyl-amidated neurotoxin. In addition, the observed molecular weight confirmed that all four disulfide bonds were intact.

The first 35 to 37 residues of curtatoxins II and III were determined by microchemical sequence analysis of the whole toxins. These toxins were highly similar, differing only at positions 9 and 14, i.e. Arg-9 and Ala-14 in curtatoxin II and Lys-9 and Phe-14 in curtatoxin III. The presence of Gln and Glu at cycle 8 assigned Asn to this position in these two toxins. Cysteine was determined by analysis of the phenylthiohydantoin-derivative of pyridylethylated cysteine. However, pyridylethylation of the nonreduced curtatoxins yielded no phenylthiohydantoin-derivative of pyridylethylated cysteine showing that the 6 cysteine residues form 4 intramolecular disulfide bonds in these toxins. Repeated Edman degradations on these two intact toxins indicated serine at cycle 38 as their carboxyl-terminal residue. To verify the carboxyl-terminal amidation of serine.3 Furthermore, the nonformylated fragments of curtatoxins II and III confirm the carboxyl-terminal acid in these samples was estimated to be as much as 20%, the origin was presumed to be from hydrolysis of the amide during sample preparation.

3 Mass accuracy in the analyses of peptides derived from curtatoxins II and III was verified by analyzing angiotensin I (M, 1298); for the curtatoxin I fragment, the mass accuracy was verified by reference to known ions from the FAB matrix. The carboxyl terminus of each component was observed to be amidated. However, curtatoxins II and III showed a noticeable contribution from the carboxyl-terminal acid as the observed signal at m/z 1296 was greater than expected from the isotope distribution of the amide at this mass. Similar distributions were also observed for the mono- and dimethylated peptides isolated after CNBr digestion in 70% formic acid (see Fig. 7). Although the presence of the carboxyl-terminal acid in these samples was estimated to be as much as 20%, the origin was presumed to be from hydrolysis of the amide during sample preparation.

4 During the original submission of this work, Skinner et al. (33) reported on the structure of the p-agatoxins (I-VI) from the funnel-web spider Agelesnops aperta. The p-agatoxin III, a minor toxin that is among the least homologous of the p-agatoxins of A. aperta, is identical to the major toxic component, curtatoxin II. Curtatoxins I and III are unique to the H. curta. Although these spiders are of a different genus they are evolutionarily related and share certain taxonomic characters.

5 C. Kristensen, personal communication.

The amino acid sequences of curtatoxins I–III.

| Peptide | Sequence |
|---------|----------|
| CT-I | S1C110V120G130T140Y150C160K170C180S190F200Y210C220H230N240H250 |
| CT-II | A1C2V3D4G5S6D7R8C9D10A11V12A13G14C15S16G17C18S19C20 |
| CT-III | A1C2V3D4G5S6D7R8C9D10A11G12G13C14S15C16H17C18S19C20 |

FIG. 8. The amino acid sequences of curtatoxins I–III.

4188.9 and 4237.4 versus 4188.6, and 4236.7 calculated from the amino acid sequences of their amidated forms (Table III). These values are consistent with carboxyl-terminal amidation and the presence of 4 intact disulfide bonds. In addition, the data also agree with the assignment of Gln over Glu at position 6 in curtatoxins II and III.

Table II compares the amino acid composition of curtatoxins II and III determined from peptide hydrolyses and amino acid sequencing. With the exception of slightly low Cys determined by amino acid analysis, the compositions determined by both methods are in excellent agreement. Notable features of curtatoxins are the high content of Cys (8), Tyr (4–5), Ser (3–5), acidic (4) and basic residues (3–4), the presence of the two dipeptide sequences Cys-Cys and Tyr-Tyr and an absence of His, Thr, and Ile.4

DISCUSSION

This study describes the first complete purification and structure of neurotoxic venom components from the funnel-web spider H. curta. Three curtatoxins were isolated by reverse-phase HPLC, anion-exchange and gel permeation chromatography. Purification was monitored with the cricket A. domestica using paralysis of the insect as the bioassay. These toxins represent the principle components in H. curta venom (see Fig. 1) responsible for irreversible paralysis and death of the insect upon envenomation. At LD50 doses (4 μg/g) of purified toxin, paralysis occurs within 30 min and death within 24–48 h. However, an LD50 dose of crude venom (24.9 μg/g) contains less than 0.1, 0.4, and 0.2 μg of curtatoxins I, II, and III, respectively, i.e. concentrations below their LD50 values. That the potency of the whole venom is greater than that of the purified curtatoxins may be explained by synergism with reversible inhibitors possibly related to the apigatoxin family which have postsynaptic effects on the glutamate receptor (5, 8), or, presence of other unidentified toxins. The amount of venom introduced by the bite of H. curta is not known and probably depends upon the type and size of the prey. However, the average amount of venom obtained from a single spider by electrophoretic milking, i.e., 0.1–0.2 μl,5 agrees with the cricket toxicity data (LD50 = 0.18 μl) for whole venom reported here.

The amino acid sequences of the curtatoxins are shown in Fig. 8. The difference in chromatographic behavior of curtatoxins II and III is attributable to the substitution of 2 amino acids at positions 9 (Lys → Arg) and 14 (Phe → Ala). The Lys6 and Phe6 substitutions increase the hydrophobic character of curtatoxin III (24) and presumably account for its increased retention time during reverse-phase and gel permeation chromatography. Curtatoxin I has 36 amino acids as...
compared to 38 for curtatoxins II and III and is less similar with deletions occurring at residues secticidal polypeptides curtatoxin I of H. curta and insectotoxin-I, has 36 amino acids with 8 cysteine residues. In this context, we have observed both similarities and differences among various arthropod toxin Cys motifs relative to those of the curtatoxins. As is shown in Figs. 9 and 10, the Cys motif and hydropathy profile of the insectotoxin-I, has 36 amino acids with 8 cysteine residues. Insect and 17. The hydropathy profiles of the curtatoxins are similar indicating a region around the Cys-Cys dipeptide that is less hydrophilic than the flanking ends of these molecules (not shown). When allowing for gaps in curtatoxin I, cysteines in all three toxins are conserved at identical sequence positions (Cys motifs) indicating highly similar folded structures. High cysteine contents in short arthropod polypeptide neurotoxins appear as a recurring theme with variation in the sequence position of these residues. In this context, we have observed both similarities and differences among various arthropod toxin Cys motifs relative to those of the curtatoxins. As is shown in Figs. 9 and 10, the Cys motif and hydropathy profile of the insectotoxin-I, has 36 amino acids with 8 cysteine residues. In a similar sequence fashion (Fig. 9). These findings suggest that these insecticidal toxins may have similar disulfide pairings and hence, folded configurations. This example may be contrasted to the different Cys motifs of the polypeptide neurotoxins with 8 cysteine residues such as the Australian funnel-web spiders Atrax robustus, Atrax versutus (26) and, the Ewing variant 3 scorpion neurotoxin Centroctes sculpturatus (27) and toxin II of the North African scorpion Androctonus australis Hector (28). Among these arthropod neurotoxins, the curtatoxins, Atrax toxins, and scorpion toxins (Fig. 9) form three distinct classes of Cys motifs. It is tempting to speculate that the different Cys motifs have a relation to the allowed molecular topologies (29) describing the stable tertiary structure of these toxins determined during the physical process of folding and disulfide formation. Moreover, the variation in folded configurations may be essential for the different receptor recognition specificities and biological modes of actions among these neurotoxins.

Of additional interest is the mechanism of the inhibitory action of the curtatoxins. Curtatoxins cause an irreversible flaccid paralysis consistent with a presynaptic blockade affecting neuromuscular function in the insect. Bowers et al. (30) reported the isolation of an irreversible presynaptic neurotoxin from H. curta venom consisting of two subunits of M, 7,000 and 9,000. Their work with abnormally excitable Droso- phila mutants indicated that inhibition of transmitter release at the motor nerve terminal was due to a specific and direct effect on presynaptic calcium channels. Jackson et al. (31) reported an irreversible inhibitor of M, 5,000 to 10,000 from H. curta. In avian cochlear nucleus neurons the apparent site of action was the postsynaptic receptor-channel complex. Entwhistle et al. (32) isolated a pure, almost neutral polypeptide of M, 5,500-5,900 from Phoneutria nigriventer. This toxin was tested on an isolated locust femur preparation and was found to generate action potentials along the length of the axone in the euronal nerve, resulting in rapid and uncontrolled twitching of the skeletal muscles. Its reported amino acid composition shows that it is not identical to the curtatoxins. The mechanism of action of these insecticidal neurotoxins from H. curta remains to be determined.

Acknowledgments—We thank Susan Treadway and Mary Lynn Points for preparing this manuscript and Steven Biedenbach for the figures. We also thank Charles Kristensen, President, Spider Pharm Inc., for providing authenticated spider venom of H. curta and A. aperta and for helpful discussions regarding this study.

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**Supplementary Section**

**Methodology**

Materials - Bovine serum was obtained from Hindustone Inc., Salt Lake City, Utah and diluted frozen at -80°C. Glycerine buffer was from Pierce and lyophilization was from Aldrich.

**High-Performance Liquid Chromatography (HPLC)** - HPLC was performed with a Waters Associates model 510 pump equipped with a Waters Associates model 441 detector and a Waters Associates model 680 integrator. The mobile phase consisted of 0.05% TFA (trifluoroacetic acid) in water. The column was packed with a Whatman Partisil-10 SAX columns. The sample was dissolved in 0.05 M potassium chloride buffer containing 0.05% TFA (trifluoroacetic acid) in water. The column was packed with a Whatman Partisil-10 SAX columns. The sample was dissolved in 0.05 M potassium chloride buffer containing 0.05% TFA (trifluoroacetic acid) in water.

**Electrophoresis** - The electrophoresis was performed using a 20% acrylamide gel, 0.1% SDS, and 0.1% Triton X-100. The gel was run at 180 V for 2 hours at 4°C. The gel was stained with Coomassie Blue R-250 and destained with 10% methanol and 5% acetic acid.

**Nuclear Magnetic Resonance (NMR)** - The NMR spectra were recorded on a Bruker AM-300 spectrometer. The samples were dissolved in D2O and the spectra were recorded at 25°C. The spectra were processed using the PD2 software.

**Mass Spectrometry** - The mass spectra were recorded using a Finnigan MAT 95 mass spectrometer. The samples were dissolved in methanol and the mass spectra were recorded in the positive ion mode.

**Circular Dichroism (CD)** - The CD spectra were recorded using a Jasco J-710 spectropolarimeter. The samples were dissolved in 0.05 M potassium chloride buffer containing 0.05% TFA (trifluoroacetic acid) in water.
TABLE I

| Curatoxin I | Curatoxin II | Curatoxin III |
|------------|-------------|---------------|
| Gly        | Phe          | Val           |
| Ala        | Asp          | Gly           |
| Ser        | Glu          | Gly           |
| Thr        | Ser          | Ser           |
| Ile        | Met          | Phe           |
| Gly        | Ala          | Glu           |
| Thr        | Thr          | Arg           |
| Ser        | Ser          | Ser           |

* Data determined on the phenylthiohydantoin derivatives of p-Cys-pCys.
* Gly quantitated as Gly + Gla.
* Cysteine (0.5 mmole), curatoxin II (1.0 mmole) and curatoxin III (0.5 mmole) were sequenced on a Beckman 110 model sequencer.

** Table II

| Amino Acid | Curatoxin II | Curatoxin III |
|------------|--------------|---------------|
| Arg        | 3.35 (±)     | 2.12 (±)      |
| His        | 1.28 (±)     | 1.33 (±)      |
| Ser        | 0.56 (±)     | 0.53 (±)      |
| Thr        | 0.53 (±)     | 0.50 (±)      |
| Val        | 1.66 (±)     | 1.81 (±)      |
| Ile        | 0.32 (±)     | 0.31 (±)      |
| Met        | 1.89 (±)     | 1.76 (±)      |
| Leu        | 1.03 (±)     | 1.02 (±)      |
| Tyr        | 0.72 (±)     | 0.72 (±)      |

* Amino acid analyses were determined by the (R)-PMB method (22). Values in parentheses are from the cysteine, methionine, arginine, and serine standards. (R)-PMB not determined.

** Table III

| Protein | Mol wt observed | Mol wt theoretical |
|---------|-----------------|--------------------|
| Curatoxin I | 4140.0 | 4202.5 |
| Curatoxin II | 4498.0 | 4508.9 |
| Curatoxin III | 6371.4 | 6279.7 |

* Results obtained from electrophoretic analysis of curatoxin II and III.

** FIG. 1

Reversal phase HPLC of whole D. hoffmanni venom. One-half ml of venom (13 mg protein) was applied to a RP-18 Hibar column equilibrated with 0.05 M Tris in 6% formic acid and washed with 0.5 M of the equilibration buffer. Phenylthiohydantoin derivatives were added with a linear gradient to 60% acetonitrile over 150 min at a flow rate of 1 ml/min. The column was eluted with a 30-min linear gradient from 0% to 100% acetonitrile. The activity-associated zones eluted in fractions 27 (curatoxin II, 15% curatoxin II) and 37 (curatoxin III) with fractions 35 and 37 comprising the major activity.

** FIG. 2

Amino-acid analysis of curatoxin II. A bioassay HPLC of curatoxin II, 5 mg protein (22), was performed on a RP-18 Hibar column equilibrated with 0.05 M Tris in 6% formic acid and washed with 0.5 M of the equilibration buffer. Phenylthiohydantoin derivatives were added with a linear gradient to 60% acetonitrile over 150 min at a flow rate of 1 ml/min. The activity-associated zones eluted in fractions 27 (curatoxin II, 15% curatoxin II) and 37 (curatoxin III) with fractions 35 and 37 comprising the major activity.
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FIG. 4. Reverse-phase chromatography of curtatoxin II. Pooled (enriched) fractions from the gel permeation step (Fig. 3) were applied to a PepRPC on 1.0/10 column equilibrated with 1% acetic acid. The column was eluted in a linear gradient from 95% acetic acid to 50% acetic acid, 2% TFA, and 98% H2O at a 1-3 ml/min flow rate. Final peaks were pooled and subjected to further analysis.

FIG. 5. Calperrin-labeled N-terminal fragments of curtatoxin II retained in the column (Fig. 3) were labeled with [35S]methionine (100 nmol) and then chromatographed on a Sephadex G-25 column equilibrated with 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.5. Fractions were assayed for radioactivity and the retention profile of the labeled peptide was determined.

FIG. 6. Reverse-phase HPLC of curtatoxin II. Chromatographic separation was performed as described in the legend to Fig. 4. The active peptide (labeled tone) eluted at 30% acetonitrile.

FIG. 7. Purification of the carboxy-terminal C-terminal fragments of curtatoxin II and III by microbore reverse-phase HPLC. The peptidylated amide toxins were degraded with CNBr (10 min at 29 °C) and then chromatographed on a Sephadex G-25 column equilibrated with 0.1 M NaCl, 0.01 M phosphate buffer, pH 7.5. The labeled fragments were eluted at 20% acetonitrile.

FIG. 8. Purification of the carboxy-terminal C-terminal fragments of curtatoxin II and III by microbore reverse-phase HPLC. The labeled fragments were eluted at 20% acetonitrile. The labeled fragments were then subjected to further purification by reverse-phase HPLC. The labeled fragments were eluted at 30% acetonitrile.
Curtatoxins. Neurotoxic insecticidal polypeptides isolated from the funnel-web spider Hololena curta.
A Stapleton, D T Blankenship, B L Ackermann, T M Chen, G W Gorder, G D Manley, M G Palfreyman, J E Coutant and A D Cardin

J. Biol. Chem. 1990, 265:2054-2059.