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Extensive multiplicity of the miscellaneous type of neurotoxins from the venom of the cobra *Naja naja naja* and structural characterization of major components

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A multiplicity of miscellaneous type neurotoxins were detected in the venom of the cobra *Naja naja naja* by use of reverse-phase HPLC and FPLC. The primary structures of major forms were determined, giving 4 novel structures. All four contain 62–65 residues, with 10 half-cystine residues and resemble the miscellaneous type of toxins from other Naja species. Differences within the species are extensive, exchanges occur at 27 positions, giving only 58% residue identity between all forms. However, the differences are largely limited to 3 regions corresponding to structurally important loops where two functional residues participating in receptor binding are exchanged. The four miscellaneous neurotoxins now characterized, together with the minor components of the miscellaneous type, the minimally four neurotoxins reported before, and other related toxins, indicate the existence of an extensive toxin gene multiplicity.

Neurotoxin; Gene multiplicity; Divergence; Isotoxin; Amino acid sequence

1. INTRODUCTION

The neurotoxins of Elapidae and Hydrophidae snake venom can be clearly distinguished into two major groups, the long (~72-residue polypeptides with 5 disulfide bridges) and short (~61 residue peptides with 4 disulfide bridges) neurotoxins [1]. In addition, some miscellaneous structures have been reported [1,2], a group of which possesses distinct features with 10 half-cystine positions [3–6], 8 of which in common with those in long neurotoxins, which have a three-loop conformation [7].

The venom of *Naja naja naja* (Pakistan) is extremely neurotoxic. Three long neurotoxins have already been characterized [8,9] from this source, together with a short neurotoxin [10]. We now report the existence of an additional group of several miscellaneous-type of neurotoxins consisting of peptides with 62–65 residues. The structures of 4 major forms were determined and show the typical half-cystine pattern in multiple components of extensive divergence within the species.

2. MATERIALS AND METHODS

Collection of crude venom and separation by C18 HPLC was carried out as described [10]. The neurotoxin fraction (C in Fig. 1 of [10]) was further purified by ion-exchange FPLC on Mono S (Pharmacia) in 20 mM sodium phosphate, pH 6.8, with a linear gradient of 0–1 M NaCl, giving 8 fractions (Fig 1). Fractions 5 and 7 were further purified by HPLC on Ultropac C18 in 0.1% TFA with a linear gradient of acetonitrile. Purified peptides were carboxymethylated with ^{14}C-labelled iodoacetate after reduction with dithiothreitol as described [10].

Digestions with Lys-C protease, Glu-C protease, and chymotrypsin were carried out at 37°C for 4 h in 0.1 M ammonium bicarbonate, pH 8.1, with an enzyme/substrate ratio of 1:50. Digestion with pepsin was performed in 5% formic acid at room temperature for 2 h with an enzyme/substrate ratio of 1:200. Resulting peptides were purified by reverse-phase HPLC on Ultropac C18 in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile.

Amino acid compositions were determined on a Beckman 121M analyzer, after hydrolysis in evacuated tubes with 6 M HCl/0.5% phenol for 24 h at 110°C. Amino acid sequence analysis was carried out with gas-phase (Applied Biosystems 470A) and solid-phase (MilliGen Prosequencer 6600) sequencers.

3. RESULTS

The separation pattern of crude venom of *Naja naja naja* yields 12 fractions thus far identified, one of which contains the neurotoxins (fraction C in Fig. 1 of [10]) and was further fractionated on Mono S to give 8 fractions (Fig. 1). Those corresponding to peaks 1–3 have already been characterized and are known to represent long and short neurotoxins [8–10], while fractions 4–8 were presently found to contain miscellaneous neurotoxins, in total 7 chromatographically different components. The final purification of major forms in

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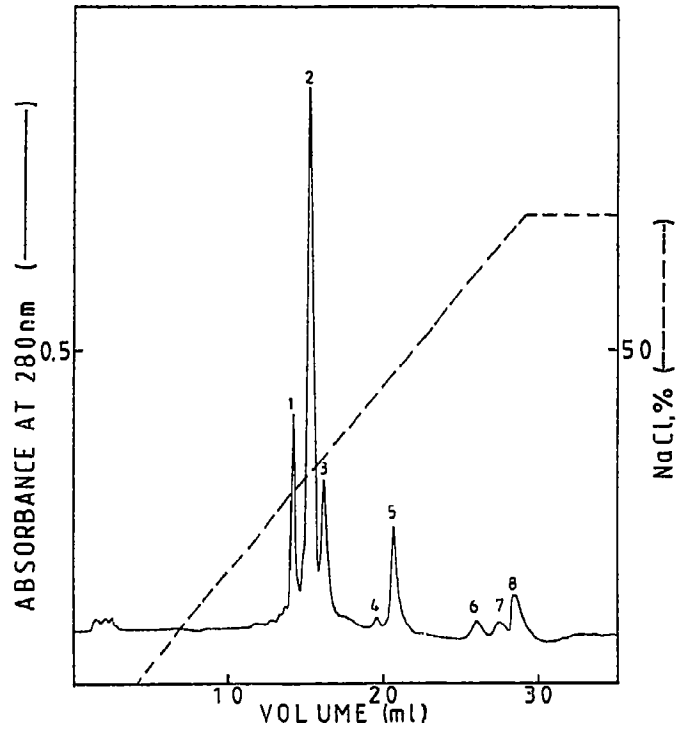

Fig. 1. FPLC fractionation of the neurotoxins from *Naja naja naja*. The neurotoxin fraction obtained by reverse-phase HPLC (fraction C in Fig. 1 of [10]) was separated on Mono S in 20 mM sodium phosphate, pH 6.8, with a linear gradient (dashed line) of 0–1 M NaCl into 8 fractions (numbered). Fractions 1–3 represent 3 long neurotoxins already reported [8–10]. Fractions 4–8 represent the miscellaneous neurotoxins.

fractions 5 and 7 was achieved by reverse-phase HPLC on Ultropac C18.

The primary structures of the major toxins in fractions 5–8 were determined as shown in Fig. 2, by sequencer degradations in gas-phase and solid-phase instruments of the intact peptides and of secondary fragments obtained by digestions with Lys-C, Glu-C, chymotrypsin and pepsin. Total compositions by hydrolysis are in agreement with the sequence data and confirm the identifications. For toxin 5, the overlap between peptides P2 and P3 was not established by sequence degradation (Fig. 2). However, a direct continuation as shown is compatible with homology with other miscellaneous-type neurotoxins [2], with compositions, and with lack of further fragments nonascribed to the whole structure.

The four toxin structures are different but have in common 10 half-cystine residues concluded to represent 5 disulfide bridges, as in other known structures of miscellaneous toxins [6]. Fig. 3 shows the structural variation among the 4 forms, and ascribes 3 gap-positions to toxin 5 in the alignment. Although the third gap coincides with a peptide bond not passed by sequencer degradation (Fig. 2), a gap at that position has also been found in other homology assignments of miscellaneous-type neurotoxins [2]. Fig. 3 also localizes the disulfide bridges and functional units in relation to the 3 loops and structurally important residues already known in long neurotoxins [7].

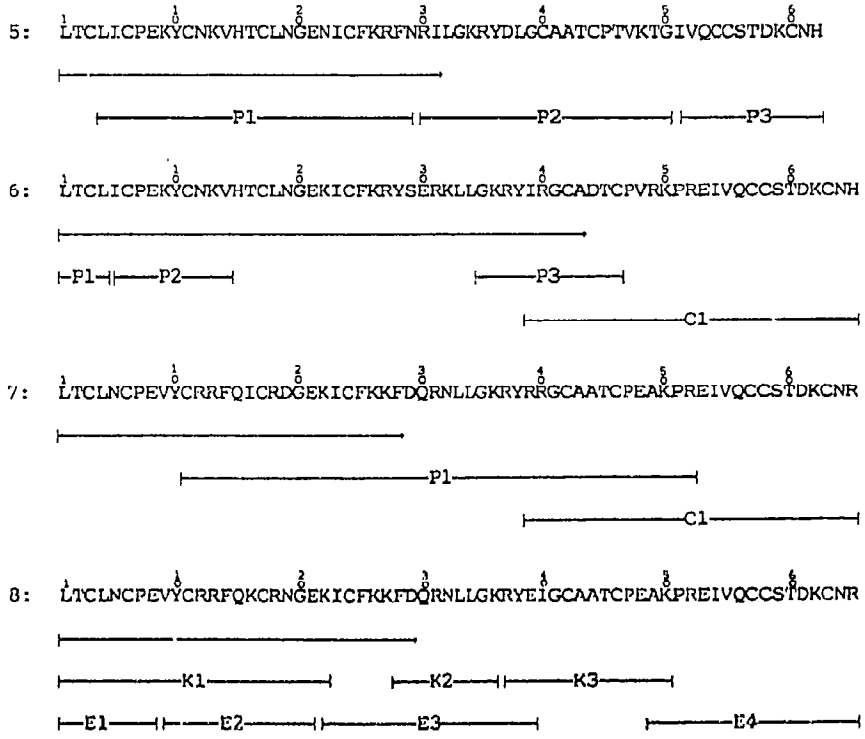

Fig. 2. Amino acid sequences of the 4 major miscellaneous neurotoxins. Lines indicate extents of sequence determination of intact peptides, lettered lines analysis of fragments obtained by digestions with pepsin (P), chymotrypsin (C), Lys-C (K) and Glu-C (E) proteases.

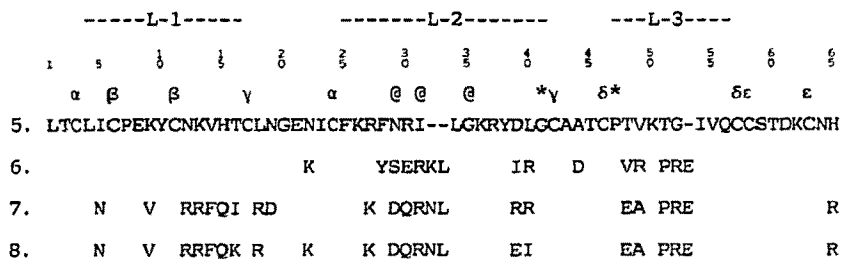

Fig. 3. Structural comparison of the 4 miscellaneous neurotoxins now analyzed from a Naja species. Top line shows toxin 5. For clarity, only residue exchanges are indicated in the remaining lines (toxins 6–8). Gap positions in toxin 5 vs the other toxins are indicated by dashes. Structurally invariant residues (*), functionally important residues (@), segments corresponding to 3 loops (lines L-1 to L-3) in long neurotoxins [7], and disulfide interconnections (Greek letters α–ϵ) corresponding to those in long neurotoxins (plus an extra disulfide, β), are indicated. Three regions of dissimilarity are visible and affect all 3 loops.

4. DISCUSSION

A group of miscellaneous-type neurotoxins has been characterized from the venom of *Naja naja naja*. The group was recovered from the major neurotoxic fraction separated by reverse-phase HPLC (Fig. 1 in [10]). The neurotoxic components of that fraction were further separated by FPLC on Mono S (Fig. 1), giving 3 long neurotoxins as major fractions and 5 minor fractions (4–8) containing the miscellaneous type of neurotoxins. Fractions 5 and 7 further showed two components in each and were separated on reverse-phase HPLC.

In the present study, the amino acid sequences of the 4 major components 5–8 were determined. They have 62–65 amino acid residues and exhibit a homology (62–83% residue identity) with the miscellaneous type of neurotoxins isolated from *Naja naja kaouthia* [6], *Naja nivea* [6], *Naja haje haje* [4], *Naja haje annulifera* [3] and *Naja melanoleuca* [5]. All these toxins consist of 62–65 residues and have 10 half-cystine residues.

X-ray diffraction studies have revealed a three-loop conformation stabilized by disulfide bridges in native neurotoxins [7]. Critical half-cystine positions are strictly conserved, and in the toxins now characterized 8 half-cystine residues (marked α, γ, δ and ϵ in Fig. 3) correspond, after insertion of a few gaps, to 4 disulfide bridges in the three-loop conformations [7]. The additional half-cystine positions in the long neurotoxins form one further bridge in the second loop, while the two additional half-cystine residues in the miscellaneous neurotoxins (marked β in Fig. 3) are concluded to form an extra disulfide bridge in the first loop.

Residue exchanges are mainly located in 3 regions, almost exactly corresponding to the loops (cf. Fig. 3). However, structurally important residues are conserved (asterisks in Fig. 3, corresponding to Gly-44 and Pro-50 in the long neurotoxins [7]). Interestingly, functionally important residues (@ in Fig. 3, including positions corresponding to Trp-29 and Asp-31 of long neurotoxins [1]) previously found to be exchanged in miscellaneous neurotoxins with high LD_{50} values [3–5] are now also exchanged, to Asp/Asn/Ser and Ile/Arg, respectively (positions 29 and 31 in Fig. 3).

The presence of the miscellaneous neurotoxins in different snake species, the similarities within the group, and the multiplicity presently shown, suggest that they may have particular functional characteristics. Like κ-bungarotoxin from *Bungarus multicinctus* [11], with a high affinity towards neuronal acetyl choline receptor, a low affinity towards nicotinic acetyl choline receptors, and a structure closely related to that of the multiple miscellaneous neurotoxins, they may possess a spectrum of specificities towards different sites. In any event, the 7 miscellaneous neurotoxins now detected, the 4 long and short neurotoxins reported earlier [8–10], and the phospholipases, protease inhibitors and cytotoxins thus far characterized [10,12–14], represent a considerable gene multiplicity for a variety of toxins in a single species.

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