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Characterization of a cytotoxin-like basic protein from the cobra (*Naja naja naja*) venom

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A cytotoxin-like basic protein has been isolated from the venom of the nominate race of cobra (*Naja naja naja* from Pakistan) by a single step of high-performance liquid chromatography. The primary structure was determined and consists of 62 amino acid residues in a single polypeptide chain. It is highly similar to that of the cytotoxin-like basic proteins isolated from other *Naja* species, but differs in two of the SS-loop structures from that of cytotoxins.

Cytotoxin-like basic polypeptide; Cobra venom; Amino acid sequence; Structure-function relationship; Homology

1. INTRODUCTION

Cytotoxins with 60–61 amino acid residues are the basic polypeptides found abundantly in the venom of Elapidae snakes belonging to the genus *Naja* [1]. Together with these major toxic polypeptides and two classes of neurotoxins, homologous cytotoxin-like basic polypeptides (CLBP) with slightly altered structure and function are also present in these venoms [2] and have been characterized from *Naja melanoleuca, N. naja annulifera, N. naja atra, N. naja*, and *N. naja siamensis* [3–8]. Their primary structures show considerable similarity with the conventional cytotoxins and more distant similarities with the short neurotoxins. CLBPs differ from cytotoxins in having an additional residue between Cys-3 and Cys-14 and in a different amino acid sequence between positions 24 and 34, lacking otherwise invariant Met residues. The half-cystine patterns are the same and suggest similar confirmations. However, CLBPs do not show neurotoxicity, and have a cytotoxicity 1–2 orders lower than that of cytotoxins, both in vivo and in vitro [5–7].

In this study, we characterize a CLBP from the venom of *N. naja naja* from Pakistan. We find a structure different from those of earlier reports. During the preparation of this manuscript a reinvestigation was also reported for two other structures [8]. Therefore, there is now complete agreement on the CLBP structures, which will be of importance for tracing the structure-function relationships of the cytotoxin membrane interactions.

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2. MATERIALS AND METHODS

Collection of the crude venom and fractionation by reverse phase high-performance liquid chromatography (HPLC) was carried out as reported [9]. Using Vydac C_{18} (The Separations Group, Hesperia, CA) and a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid, CLBP is eluted as a minor fraction in between the two major cytotoxin fractions. The homogeneity and the molecular weight were estimated by SDS/polyacrylamide gel electrophoresis. Carboxymethylation with ^{14}C-labelled iodoacetate was performed after reduction with dithiothreitol [10].

Different enzymatic digestions were carried out in 0.1 M ammonium bicarbonate, pH 8.1, with chymotrypsin, Asp-specific protease and Glu-specific protease at enzyme to substrate ratios of 1:100, 1:50, and 1:25, respectively [10]. Digests were separated by HPLC on Ultropak C_{18} (LKB) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile [10]. The peptides were structurally analyzed with an Applied Biosystems 470A gas phase sequencer using phenylthiohydantoin identification by HPLC as described [11]. The intact peptide was also analyzed manually with the DABITC method [12] and on a Beckman 890C liquid phase sequencer. Positions of carboxymethylcysteine residues were confirmed by monitoring the radioactivity of each cycle. Amino acid compositions were determined with a Beckman 121M analyzer after hydrolysis in evacuated tubes for 24 h at 110°C with 6 M HCl/0.5% phenol.

3. RESULTS

Separation on reverse-phase HPLC with acetonitrile in 0.1% trifluoroacetic acid gives over 10 components from crude venom of *N. naja naja* [9], with CLBP in between the two major cytotoxins. The material corresponding to CLBP was homogeneous on SDS/PAGE, and by sequence analysis.

The complete primary structure of CLBP was determined by liquid-phase sequencer analysis of the intact polypeptide and by gas-phase sequencer analysis of overlapping peptides from different enzymatic digests (fig.1). The structure shows 62 amino acid residues in a

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Table 1

Total composition of the CLBP from *N. naja naja*

Residues	mol/mol	
Cys (Cm)	8.0	(8)
Asp	6.7	(2)
Asn		(5)
Thr	3.2	(3)
Ser	2.0	(2)
Glu	1.6	(1)
Gln		(1)
Pro	5.0	(5)
Gly	2.1	(2)
Ala	2.9	(3)
Val	3.6	(2)
Met	0	(0)
Ile	1	(1)
Leu	6.7	(7)
Tyr	2.0	(2)
Phe	3.0	(4)
His	1.0	(1)
Lys	11.0	(11)
Arg	1.2	(1)
Trp	0	(0)
Sum		62

Values in molar ratios, and within parentheses from sequence analyses

single chain with 8 half-cystine residues as given in fig.1. Amino acid analyses of the carboxymethylated polypeptide (table 1) and the enzymatic fragments are in full agreement with the sequence determined.

4. DISCUSSION

Characterizations of several CLBPs from various snake species reveal that the CLBP primary structure is conserved and closely similar to those of the typical cytotoxins and short-chain neurotoxins [5]. However, a discrepancy of previous data at a few positions has been noticed and was recently clarified [8]. That report was published after assembly of the present results and our work shows that *N. naja naja* from Pakistan also supports the recently revised CLBP structures.

The primary structure was now established from CLBP isolated to homogeneity by a single step of reverse-phase HPLC. The structure differs only at two positions from the CLBP of *N. naja siamensis* [7] and at no position from the recently reported *N. naja atra* and *N. naja* structures [8].

Since the CLBP half-cystine pattern is the same as in cytotoxins, the conformations are likely to be highly similar. Therefore, by fitting the present CLBP sequence in the frame of cytotoxins [13], it is clear that among the three loops of the structure, the first loop is extended by one amino acid residue (His-4 in fig.1), the central loop has major differences between positions 24–30, including the totally different part lacking the

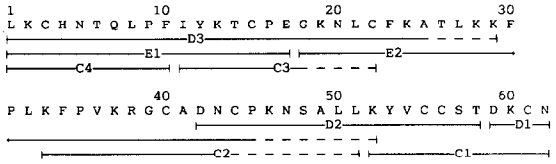

Fig.1. Primary structure of a CLBP from *N. naja naja*. D denotes peptides from the cleavage with the Asp-specific protease, E those from that with the Glu-specific protease, and C those from that with chymotrypsin. Subsequent numbers show elution orders of peptides in each digest from the reverse-phase HPLC peptide purification.

otherwise invariant Met residues (at positions corresponding to Ala-25 and Leu-27 in fig.1), while the third loop does not appear to show any significant differences.

Since the mechanism of cytotoxin and cell membrane interaction has only been speculated upon based on charged and hydrophobic residues [14,15], the differences in the correct CLBP structures as reported now and in [8], together with the low CLBP cytotoxicity, might be helpful for the understanding of the interactions of cytotoxins with cell membranes.

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