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Cobra venom contains a pool of cysteine-rich secretory proteins

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Abstract

A large family of cysteine-rich secretory proteins (CRISPs) includes proteins of different origin, the function of the majority of CRISPs being unknown. For CRISPs isolated from snake venom, two types of activities were found: two proteins blocked cyclic nucleotide-gated ion channels, several others blocked potassium-stimulated smooth muscle contraction. Thus, snake CRISPs represent potentially valuable tools for studies of ion channels, which makes promising a search for new CRISPs. Here we report on the isolation of several novel CRISPs from the venoms of Asian cobra *Naja kaouthia* and African cobra *Naja haje* using a combination of different types of liquid chromatography. Four CRISP variants were identified in *N. kaouthia* venom and three proteins, one of them acidic, were found in *N. haje* venom. Acidic CRISP was found in a reptilian venom for the first time. Our data suggest that each cobra venom contains a pool of different CRISPs.

Keywords: Cobra venom; Cysteine-rich secretory proteins; Isolation; Properties

A family of cysteine-rich secretory proteins (CRISPs) is a group of secreted single-chain proteins with molecular weights of about 20–30 kDa and homologous amino acid sequences comprising 16 conserved cysteine residues which form 8 disulfide bonds. Fourteen cysteine residues are located within the C-terminal half, 10 of them being within the C-terminal third of the polypeptide chain. However, the N-terminus is more conserved as compared to other regions of these proteins.

CRISPs have been found in secretions of mammalian exocrine glands, first of all in male reproductive tracts [1], in human granulocytes and plasma [2], and in some organisms of other taxons (for references see [3]), including reptilians. The reptilian CRISPs were found mainly in their venoms.

The first described reptilian CRISP was helothermine from the skin secret of the lizard *Heloderma horridum horridum* [4]. Later on a number of CRISPs were isolated from the venoms of snakes belonging to different families and inhabiting different continents. Thus, CRVP (cysteine-rich venom protein) from *Trimeresurus mucrosquamatus* (*Viperidae*, Asia) [5], pseudechetoxin from *Pseudechis australis* (*Elapidae*, Australia) [6], protein 25k from *Naja kaouthia* (*Elapidae*, Asia) [7], latisemin from sea snake *Laticauda semifasciata* (*Hydrophidae*), and tigrin from *Rhabdophis tigrinus tigrinus* (*Colubridae*) [8] have been described. Recently, the list of Asian Elapid CRISPs has been extended by ophanin from *Ophiophagus hannah* [9], bucarin from *Bungarus candidus* (Accession #P81993 in SwissProt), and by two proteins from *Naja atra*, CRVP1 and CRVP2 [10]. Protein 25k and CRVP1 represent probably one and the same protein as sequences of protein 25k fragments comprising 101 residues, determined by peptide sequencing, coincide completely with corresponding fragments of CRVP1 amino acid sequence deduced from cDNA.

All these snake venom proteins consist of a single polypeptide chain with a molecular mass of 23–26 kDa. They are basic in nature and contain 8 disulfide bonds.

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The list of Elapid CRISPs seems to be far from completion. Thus, cross-reactivity screening of *Elapidae* venoms with antiserum to triflin, a CRISP from the venom of *Trimeresurus flavoviridis* (*Viperidae*), detected the presence of similar antigens in venoms of *Hemachatus hemachatus*, *Naja mocanbigue*, *Naja nivea*, and of two antigens in *N. haje* venom [9]. Yamazaki and Morita [3] communicated on the presence of two CRISPs in *N. kaouthia* venom, but did not give any details on them. Here we report on the isolation of two novel CRISPs from the cobra *N. kaouthia* venom and three CRISPs from the African cobra *N. haje* venom, one of the *N. haje* proteins being acidic.

Materials and methods

Naja kaouthia and *Naja haje* venoms were obtained from living cobras kept in captivity as described in [11]. All other reagents were of the highest purity commercially available.

Isolation and purification of proteins

Eight hundred milligrams of dried venom was dissolved in 1 ml of 0.1 M ammonium acetate buffer, pH 6.2, and applied onto a column (4.5 × 150 cm) with Sephadex G50 sf (Amersham Biosciences) equilibrated with the same buffer. Fractions I–IV were pooled as depicted in Figs. 1A and B and lyophilized. Fraction II *N. kaouthia* was separated on a HEMA BIO 1000 CM column (8 × 250 mm, Tessek, Czech Republic) in an ammonium acetate gradient from 5 to 600 mM (pH 7.5) in 60 min at a flow rate of 1.4 ml/min. Separation of fraction II *N. haje* was performed on the same column with the following program: isocratic elution in 5 mM ammonium acetate (pH 7.5) for 5 min, then linear gradient from 5 to 140 mM in 14 min and from 140 to 180 mM in 20 min, followed by two more gradients: from 180 to 360 mM in 11 min and from 360 to 400 mM in 20 min, and final isocratic elution in 400 mM ammonium acetate for 5 min.

Fractions II-6, II-10, and II-11 of *N. kaouthia* venom as well as II-4 and II-7 of *N. haje* venom were lyophilized and further separated on a Vydac C18 column (4.6 × 250 mm) in an acetonitrile gradient from 15 to 45% in 30 min in the presence of 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fraction II-1 of *N. haje* venom from the cation-exchange column was separated on a HEMA BIO1000 DEAE column (8 × 250 mm, Tessek, Czech Republic) in an ammonium acetate gradient from 5 to 400 mM (pH 7.5) in 40 min at a flow rate of 0.5 ml/min.

Electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (PAGE–SDS) both under reducing and non-reducing conditions was performed according to [12].

Pyridylethylation, Edman degradation, and recording of matrix-assisted laser desorption ionization (MALDI) mass spectra were performed as described earlier [11]. An alignment and comparison of determined N-terminal amino acid sequences to those of known proteins were performed using program BLASTP 2.2.5 at ExPASy molecular biology server ([13]; http://us.expasy.org/cgi-bin/blast.pl).

Fluorescence spectra were recorded in 50 mM Tris–HCl buffer, pH 7.4, on Hitachi F-4000 spectrofluorimeter.

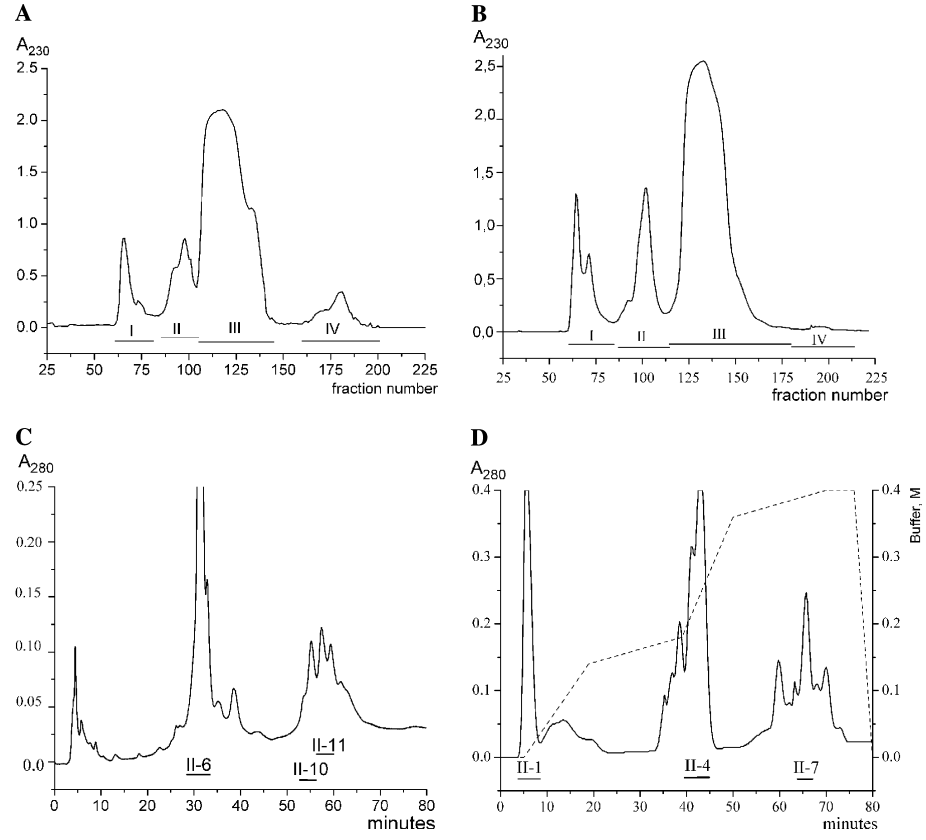

Fig. 1. Separation of crude venoms of *N. kaouthia* (A) and *N. haje* (B) by gel filtration on Sephadex G-50sf column (4.5 × 150 cm) in 0.1 M ammonium acetate buffer, pH 6.2, fraction volume 9 ml. Separation of fractions II of *N. kaouthia* (C) and *N. haje* (D) by cation-exchange HPLC on a HEMA BIO 1000 CM column in a gradient of ammonium acetate concentration (pH 7.5).