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Characterization of phospholipase A2 from the venom of Horned viper (*Cerastes cerastes*)

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Phospholipase A2 has been purified from the venom of Horned viper (*Cerastes cerastes*) by gel permeation chromatography followed by reverse-phase HPLC. The primary structure was established by sequence analysis of the intact protein and its enzymic peptides. The structure has 120 residues, properties like other group IIB phospholipases, but only 45–55% identity with the enzyme from other viperid species, and large variations even within the species (26% residue differences at known positions in another form).

Phospholipase A2; Amino acid sequence; Venom; Isozyme; Intra-species variation; Strain differences

1. INTRODUCTION

Phospholipase A2 is common in mammalian pancreas and in the venom of snakes. The biochemical and pharmacological actions of the venom enzyme have been extensively studied [1,2] and the structures are known from many sources [3]. Recently, we reported the structure of phospholipase A2 from the cobra *Naja naja naja* and noticed large variations [4].

We have now isolated phospholipase A2 from the venom of the Horned viper (*Cerastes cerastes*) of south-western Pakistan. The primary structure has been determined and compared with that of other phospholipases A2 from viperid snakes. Large variations are found both within and between species.

2. MATERIALS AND METHODS

The snakes were collected from south-west (Baluchistan) Pakistan (with the permission of the Health Ministry) and identified by the Zoological Survey Department, Karachi. The venom was extracted by pressing the poison gland in deionized water. It was then recovered by lyophilization and immediately stored at −30°C.

The crude venom (~200 mg in 5% acetic acid) was fractionated on Sephadex G-50 (Pharmacia) in 5% acetic acid. Fraction 3, containing phospholipase A2, was further purified by reverse-phase HPLC on Vydac C18 (Phenomenox, New York) in 0.1% aqueous trifluoroacetic acid with a linear gradient of acetonitrile. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [5].

The protein was reduced with dithiothreitol, carboxymethylated with ^{14}C-labelled iodoacetate [4] and cleaved in separate batches with *Achromobacter* Lys-C protease, staphylococcal Glu-C protease, and *Pseudomonas* Asp-N protease, all in 0.1 M ammonium bicarbonate, pH 8.1, at enzyme/substrate ratios of 1:50. Peptides were separated by reverse-phase HPLC on Ultropac C18 in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile [4].

Total compositions were determined with a Beckman 121M amino acid analyzer after acid hydrolysis for 24 h with 6 M HCl containing 0.5% phenol in evacuated tubes at 110°C. Amino acid sequences of the intact protein and the peptide fragments were analyzed with an ABI 470A gas phase sequencer.

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3. RESULTS

The crude venom was separated on Sephadex G-50 into five major fractions (Fig. 1, top). The material corresponding to peak 3 was further separated by reverse-phase HPLC on Vydac C-18 into one major and six minor fractions (Fig. 1, bottom). Peak 5 from the reverse-phase HPLC corresponds to a homogeneous band on SDS-polyacrylamide gel electrophoresis, with an apparent molecular mass of 14 kDa. The primary structure was elucidated by sequence analysis of the intact protein and the peptides from digests with endoproteases Lys-C, Glu-C and Asp-N. The peptides from these enzymatic digestions were purified by HPLC on Ultropac C18. The amino acid compositions of the intact protein (Table I) and the peptides are in agreement with the compositions from sequence analysis. The primary structure is shown in Fig. 2 (top).

4. DISCUSSION

The primary structure of Horned viper phospholipase A2 (Fig. 2) has 120 residues with conserved residues relative to six pairs of half-cystine residues typical of group IIB phospholipases A2 [6] and an apparently free thiol group.

The sequence around His-47, i.e. the segment 41–53

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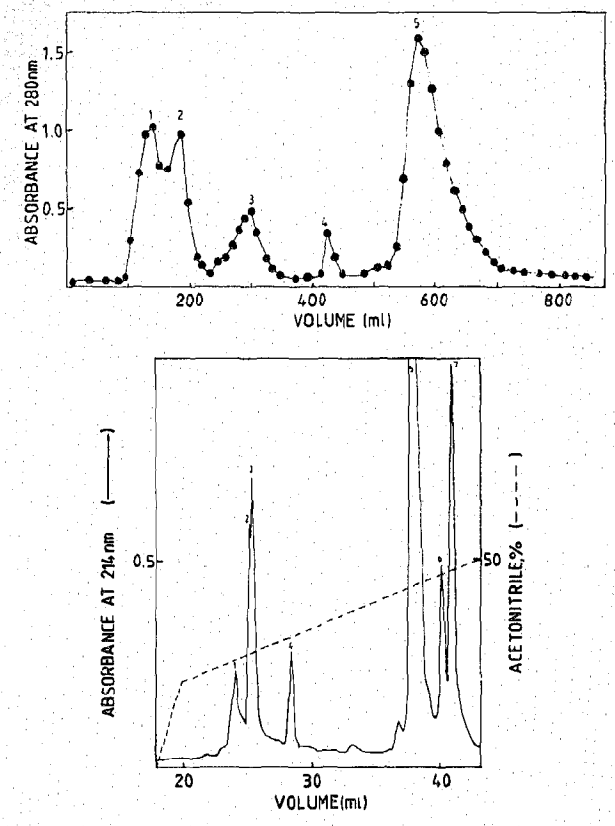

Fig. 1. Purification of *Cerastes* phospholipase A2. Chromatography of crude venom on Sephadex G-50 superfine (2.5 × 80 cm) in 5% acetic acid (top) and HPLC (bottom) of the major phospholipase peak (3 in top panel) on Vydac C18 in 0.1% trifluoroacetic acid with a gradient of acetonitrile as shown. Peak 5 (bottom panel) represents the phospholipase analyzed.

Table I

Total composition of Horned viper phospholipase A2

Residue	Acid hydrolysis	Sum of sequence determination
Cys	12.3	13
Asp	16.2	9
Asn		7
Thr	7.8	8
Ser	6.5	7
Glu	10.0	4
Gln		5
Pro	3.7	4
Gly	11.4	12
Ala	4.2	4
Val	6.8	7
Met	1.9	2
Ile	3.4	4
Leu	2.2	2
Tyr	9.9	10
Phe	5.9	6
Trp	0.6	1
Lys	9.2	9
His	1.1	1
Arg	5.2	5
Sum		120

Values given are molar ratios after acid hydrolysis for 24 h and from the sum of sequence analysis. Trp analyzed after hydrolysis with methane sulphonic acid

involved in the catalytic activity [7], is similar to other viperid group IIB enzymes [6] with the exception of residue 53, where Arg replaces Lys as in *Vipera ammodytes* [7]. The homology with phospholipases A2 of *Bitis caudalis*, Horned viper (Caudoxin), *Bitis gabonica*, *Crotalus atrox* and *Vipera ammodytes* is fairly distant (45–55% residue identity). The conserved residues are clustered at positions 24–32, 40–52 and 86–96 (present numbering system; Fig. 2). Only 38% of the structure is conserved in all these species (46 posi-

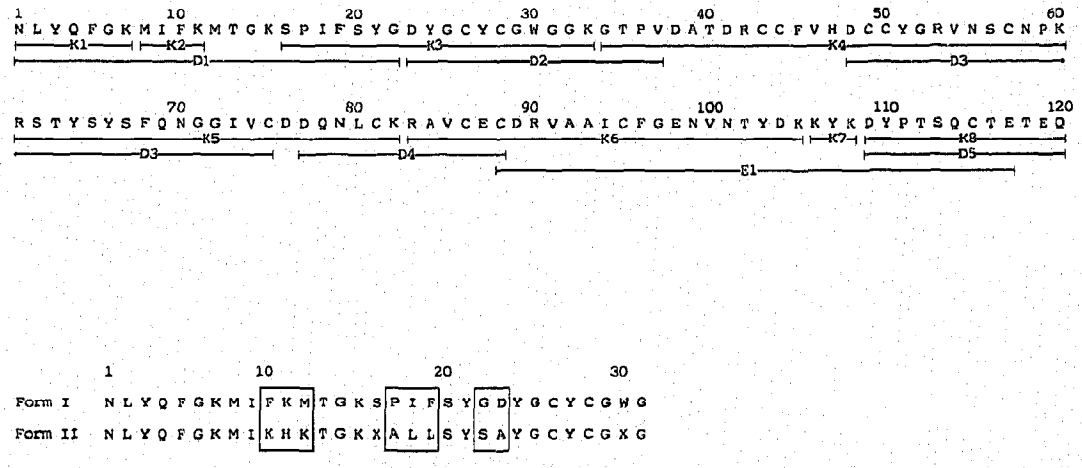

Fig. 2. Primary structure of Horned viper (*Cerastes cerastes*) phospholipase A2 and the positions of peptides analyzed (K1–K8 for peptides from Lys-C cleavage, D1–D5 from Asp-N cleavage and E1 from Glu-C cleavage). A large variation within the species is illustrated by comparisons (bottom) with the venom phospholipase A2 from the same species of African origin, revealing 8 differences for 31 positions (26%) analyzed [8]. Residue differences are boxed. Form I (this work) and Form II [8] refer to the two types (isozyme or strain differences) known within the species, the major form of Asian origin now characterized, and the one isolated from venom of African origin.

tions). Interestingly, variations are considerable even within the species. We have previously noticed this regarding sub-species of *Naja naja* [4] and now find a large variation also within *Cerastes cerastes*. Thus, an N-terminal part of a phospholipase A2 of this species from Tunisia rather than Pakistan was recently reported [8]. The two phospholipase A2 forms thus analyzed from one species vary at 8 (26%) of 31 positions reported for the African form (cf. Fig. 2, bottom). Similarly, large isozyme variations have been noticed between phospholipases A2 in the Japanese Habu snake [9], suggesting that intra-species isozyme or strain variations are extensive for this enzyme.

Functionally, the region between positions 53 and 76 has been assumed to determine the anticoagulant activity of phospholipase A2 [10]. This region is positively charged in potent phospholipases A2, but negatively charged in weak and non-anticoagulant phospholipases A2. In the *Cerastes* enzyme now analyzed this region is positively charged, containing three basic residues at positions 53, 60 and 61 (cf. Fig. 2). Similarly, the sequence from residues 79–87 with the pattern 000+ +000− (NLCKRAVCE) is close the pattern 000+ + +00− (NACKKKVCE) in caudoxin required for myotoxicity [3], indicating that the enzyme is weakly myotoxic. Thus, it is concluded that the *Cerastes* enzyme has the regions which correspond to the anticoagulant and myotoxic activities.

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