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Purification and characterization of a Kunitz-type trypsin inhibitor from Leaf-nosed viper venom

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A Kunitz-type trypsin inhibitor was purified from Leaf-nosed viper venom and the primary structure determined by peptide analysis. In relation to other trypsin inhibitors, the protein has an extended C-terminal segment and a distinct pattern of residue alterations at the functionally important contact sites with proteases.

Trypsin inhibitor; Amino acid sequence; C-terminal extension; Contact site; Homology

1. INTRODUCTION

Snake venom contains serine protease inhibitors in addition to enzymes and toxins. Several of these inhibitors have been characterized from venoms of viperid and elapid snakes [1–4]. These inhibitors belong to the Kunitz-type trypsin inhibitor family present also in pancreas [5]. Knowledge of primary structures of several members of the family is important for further understanding of the interactions with serine proteases, since a number of variations within the contact areas with trypsin and related enzymes has been found [6].

The present report describes the purification and characterization of Kunitz-type trypsin inhibitor from Leaf-nosed viper venom and demonstrates structural variations in the protease contact sites.

2. MATERIALS AND METHODS

Crude venom of Leaf-nosed viper was fractionated by Sephadex G-50 chromatography [7]. Fraction 2 (cf. Fig. 1 in [7]) containing inhibitor was further purified by reverse-phase HPLC on Vydac C_{18} (Phenomenex, New York) with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid. The material corresponding to peak 2 from this step was separated on Mono-S FPLC (Pharmacia) in 50 mM ammonium acetate, pH 4.8, with a linear gradient of 0–0.5 M NaCl. The Pharmacia Phast system was used for SDS-polyacrylamide gel electrophoresis and Coomassie Phast blue R staining.

The protein was reduced with dithiothreitol, carboxymethylated with ^{14}C-labelled iodoacetate [7] and cleaved in separate batches with Lys-C protease, and chymotrypsin, both in 0.1 M ammonium bicarbonate (pH 8.1) at 37°C for 4 h with protease/substrate ratios of 1:50. Total compositions were determined with an LKB 4151 Alpha plus amino acid analyzer after acid hydrolysis for 24 h in evacuated tubes at 110°C with 6 M HCl containing 0.5% phenol. Amino acid sequences were determined with an ABI 470A gas phase sequencer.

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3. RESULTS

The separation of Leaf-nosed viper crude venom into 5 fractions by Sephadex G-50 chromatography has been described [7]. The second fraction was now separated by reverse-phase HPLC (Fig. 1A) and material corresponding to peak 2 was purified further by FPLC on Mono-S (Fig. 1B). Peak 5 of the Mono-S chromatogram corresponded to a homogeneous band on SDS-polyacrylamide gel electrophoresis at an apparent molecular mass of about 6.5 kDa. The primary structure of this material was established by sequence analysis of

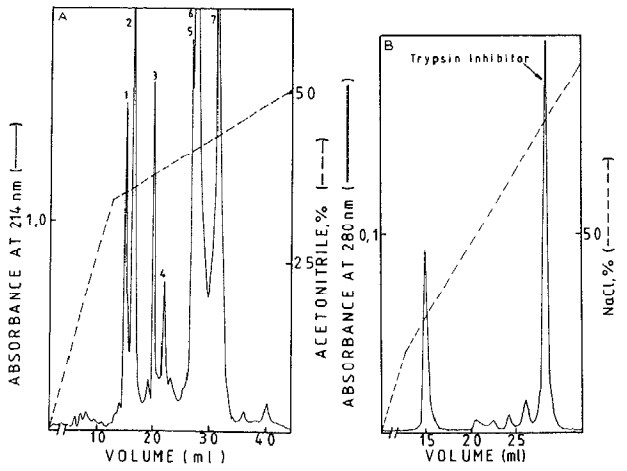

Fig. 1. Purification of Leaf-nosed viper trypsin inhibitor. (A) HPLC on Vydac C_{18} in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile; starting material: Sephadex G-50 fractionated venom, fraction 2 [7]. (B) FPLC on Mono-S in 50 mM ammonium acetate with a linear gradient of NaCl; starting material corresponding to peak as indicated in (A).

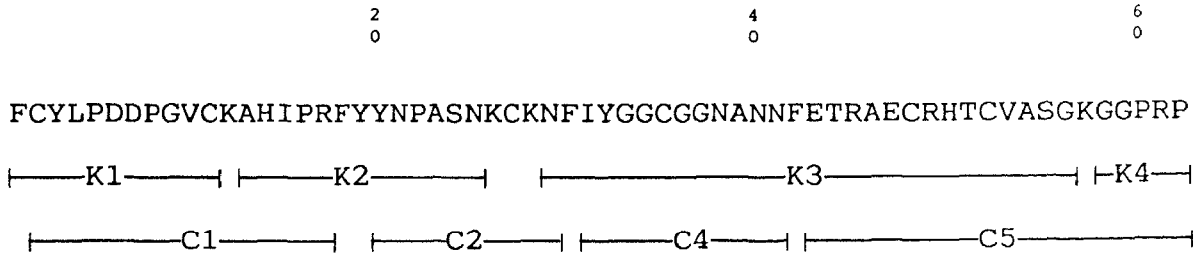

Fig. 2. Primary structure of Leaf-nosed viper trypsin inhibitor and the positions of peptides analyzed. K (K1–K4) indicates peptides from Lys-C cleavage and C (C1–C5) represents those from chymotrypsin cleavage.

peptides from separate digests with Lys-C protease and chymotrypsin. The amino acid compositions of the intact protein (Table I) and peptides are in agreement with the compositions from sequence analysis (Fig. 2).

The structure determined is homologous, with respect to both amino acid sequence and positions of Cys residues, to Kunitz-type protease inhibitors characterized from venom and other sources.

4. DISCUSSION

The Kunitz inhibitor was isolated from Leaf-nosed viper venom by a combination of gel filtration, reverse-phase HPLC and cation exchange FPLC. The 62 primary structure has six Cys residues. The Cys pattern and the entire-chain homology identify the venom component as a Kunitz-type inhibitor. A feature unlike that for most of the other many characterized trypsin inhibitors is a 6 residue extended C-terminal segment (Fig. 3). Interestingly, however, a similar pattern of C-terminal extension has been observed also for the chymotrypsin inhibitor of the venom of the closely related species Long-nosed viper [3]. Other molecules with extended C-termini from non-venom sources are represented by protease inhibitors from bovine colostrum [8] and turtle egg white [9]. The structural or functional role of the extended C-terminal segment is not known.

X-Ray crystallographic studies of Kunitz-type protease inhibitors have resulted in the characterization of two contact sites with proteases, a main one and a weak one [10]. The specificity of the inhibitor towards proteases has been found to be associated with the main contact site. Thus, a basic residue, Lys/Arg, at position 15 (homology number in Fig. 3) within the main contact site is considered to be essential for trypsin inhibitor activity [4]. In contrast, residues like Phe, Leu and Tyr at this position are typical for chymotrypsin inhibitors [11]. The presence of Lys at this position, thus identifies the polypeptide chain as a Kunitz-type trypsin inhibitor. Known relationship for the basic pancreatic trypsin inhibitor and other inhibitors within this family now allow further functional conclusions on protease interacting residues at critical positions [12].

The residues involved in the main contact site and at the region of the weak contact site with serine proteases are well conserved (Fig. 3). The only exchange, at the main contact site, is a hydrophobic residue Val-13, instead of a basic residue, Arg in closely related viper venom inhibitors (Fig. 3). Notably, a similar exchange was also observed in the trypsin inhibitor characterized from the elapid snake *Naja naja naja* [4], where Arg is exchanged for Leu as shown in Fig. 3.

The residue identity between this polypeptide chain and those of the group of other venom inhibitors is considerably higher than that between the present inhibitor and the phylogenetically distant bovine inhibitor (Fig. 3). The most extensive similarity was found with the trypsin inhibitor of a closely related species, Long-nosed viper (72% residue identity) [2]. The main and weak contact sites of this inhibitor are identical to those of the Long-nosed viper except for one exchange at both sites, i.e. Arg-13 at the main contact site and Pro-39 at the weak contact site. The residue identity with trypsin inhibitor of bovine pancreas, *Naja naja naja*, and Russel's viper is fairly distant (43–60%).

Table I

Total composition of the trypsin inhibitor

Residue	Acid hydrolysis	Sum of sequence
Cys	5.7	6
Asp + Asn	8.1	2 + 6
Thr	1.5	2
Ser	1.7	2
Glu + Gln	2.3	2 + 0
Pro	6.2	6
Gly	8.4	8
Ala	4.8	5
Val	1.6	2
Ile	1.8	2
Leu	1.1	1
Tyr	4.2	4
Phe	3.9	4
Lys	4.3	5
His	1.5	2
Arg	4.1	4
Sum		62

Values represent molar ratios after acid hydrolysis and sequence analysis. Cys analyzed as Cys(Cm).

Fig. 3. Comparison of amino acid sequence of the trypsin inhibitor from Leaf-nosed viper. Trypsin inhibitor now characterized (top line) is aligned with trypsin inhibitors from Long-nosed viper [2], Russel's viper [1], *Naja naja naja* [4] and bovine pancreas, [13] as listed 2–5. Residues at the main and the weak contact sites of the inhibitor with proteases are indicated by @ and asteriks, respectively. Sequence homology numbers are given according to the sequence of bovine pancreatic trypsin inhibitor [13].

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