Purification and characterization of a chymotrypsin Kunitz inhibitor type of polypeptide from the venom of cobra (*Naja naja naja*)

Jawed Shafqat[2], Zafar H. Zaidi[2] and Hans Jörnvall[1]

[1] *Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden* and [2] *HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan*

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A chymotrypsin Kunitz inhibitor type of polypeptide has been isolated form the venom of *Naja naja naja* by reverse phase HPLC and cation exchange FPLC. It is present in a considerably lower amount than that of the corresponding trypsin inhibitor. The primary structure, determined by sequence analysis of the whole molecule and its tryptic peptides, has 57 residues with an apparent molecular mass of 6.2 kDa. The main contact site with the protease (P1) has a Phe, showing the specificity of the inhibitor. Of residues considered functionally important in Kunitz-type inhibitors, Gly-36 is replaced by Ser in a segment of weak contacts with the protease.

Snake venom; Cobra; HPLC-FPLC separation; Kunitz-type chymotrypsin inhibitor; Amino acid sequence; Weak contact site replacement

1. INTRODUCTION

The presence of Kunitz-type serine protease inhibitors in the venoms of Elapidae and Viperidae snakes is well-known [1] and several of these inhibitors have been characterized [2–6]. They are non-toxic, basic polypeptides. Their actual role in the venom has not been directly established, but their inhibitory properties on regulatory mechanisms in tissues, influencing the proteases of coagulation, fibrinolysis and inflammation, have of course been considered. In a few cases, the inhibitors have been observed to give a synergistic increase in the toxicity of some toxins [7]. During our investigation of components of the venom from *Naja naja naja* (Pakistan), we have reported on the presence of a highly potent, Kunitz-type trypsin inhibitor, having K_i values of 3.5×10^{-12} M [6]. We have now also isolated a chymotrypsin inhibitor-like polypeptide from the cobra venom. Determination of its primary structure clearly identifies it as a Kunitz-type inhibitor.

2. MATERIALS AND METHODS

Crude cobra venom was separated by reverse phase HPLC as described [6,8]. The inhibitor eluted immediately after the neurotoxin as a small fraction (peak D in Fig. 1 of [6]). It was purified further by FPLC on Mono S in 20 mM sodium phosphate, pH 6.8, with a linear gradient of 0–1 M NaCl, and was then re-chromatographed by the reverse phase HPLC step. The pure inhibitor (6 nmol) was reduced with dithioerythritol and carboxymethylated with ^{14}C-labelled iodoacetate [6]. Digestion with trypsin was carried out in 0.1 M ammonium bicarbonate, pH 8.1, at 37°C for 4 h with an enzyme/substrate ratio of 1:100, by weight. The tryptic peptides were separated by reverse phase HPLC on C18 (Ultropak, LKB) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile [6].

Amino acids were determined with a Beckman 121M analyzer after acid hydrolysis at 110°C in 6 M HCl/0.5% phenol for 24 h in evacuated tubes. Amino acid sequences were analyzed with an Applied Biosystems 470A gas-phase sequencer and a Hewlett-Packard HPLC for thiohydantoin identification [9] or a MilliGen ProSequencer 6600 solid-phase instrument utilizing arylamine membranes for C-terminal attachment. The carboxymethylcysteine positions were confirmed by monitoring the radioactivity.

3. RESULTS

The separation pattern of cobra (*Naja naja naja*) crude venom upon reverse phase HPLC on Vydac C18 has been reported [6]. The present inhibitor corresponds to one of the minor fractions (D in Fig. 1 of [6]). It was further purified by FPLC on Mono-S (Fig. 1), eluting early (peak 1 in Fig. 1) and being separated from a contaminating neurotoxin derived from the adjacent fraction of the previous HPLC step (peak C in [6]).

The inhibitor was reduced, ^{14}C-carboxymethylated and analyzed. The amino acid sequence was obtained by solid-phase sequencer (MilliGen 6600) degradation of the intact molecule utilizing C-terminal attachment, complemented by gas-phase sequencer degradation (Applied Biosystems 470A) of the tryptic peptides for establishment of the C-terminal end and verification of

Correspondence address: H. Jörnvall, Department of Chemistry I, Karolinska Institutet, S-104 01 Stockholm, Sweden

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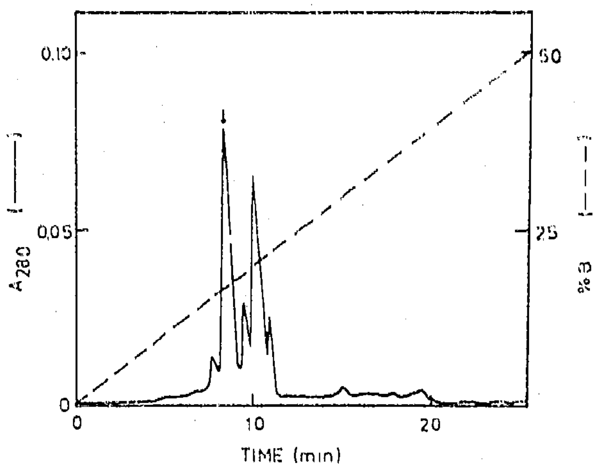

Fig. 1. FPLC separation on Mono-S in 20 mM sodium phosphate, pH 6.8, with a linear gradient of NaCl (solution B is 1 M NaCl in the buffer). Material applied: from previous step of HPLC (corresponding to peak D in [6]). The first peak (at the arrow) represents the inhibitor, the second the contaminating neurotoxin.

Table I

Total composition of chymotrypsin inhibitor.

Residues	(mol/mol)	
Cys	6.2	(6)
Asp / Asn	6.2	(1) / (5)
Thr	4.0	(4)
Ser	6.7	(7)
Glu / Gln	2.4	(2) / (0)
Pro	2.4	(2)
Gly	4.4	(4)
Ala	3.1	(3)
Val	2.6	(3)
Ile	1.3	(1)
Leu	1.1	(1)
Tyr	4.7	(5)
Phe	4.9	(5)
Lys	0.8	(1)
His	1.0	(1)
Arg	5.6	(6)
Sum		57

Values from acid hydrolysis, and, within parentheses, sequence analysis.

internal regions (Fig. 2). The total composition (Table I) supports the structure deduced.

The results identify the protein as a Kunitz-type protease inhibitor. Thus, it is distantly homologous (58% residue identity) to the trypsin inhibitor in the venom of the same species [6], but in contrast to the latter, it has Phe at position 15 (Fig. 3). This is the specificity-determining position, P1 of inhibitors [10], and suggests that the present venom molecule is of the chymotrypsin inhibitor type. It is present in much lower amounts than the corresponding trypsin inhibitor (roughly 1/10, as judged from the relative peak heights upon purification, cf. peaks D and B in [6]), and appears not to have been characterized before in Elapidae snakes, but is known from a Viperidae snake [11] and silkworm larvae [12]. The structures of all these molecules and bovine basic pancreatic trypsin inhibitor [13] are compared in Fig. 3, showing the distant similarities, the distinguishing P1 positions of the two groups, the important residues in common, and the deviating functional residues now detected, as discussed below.

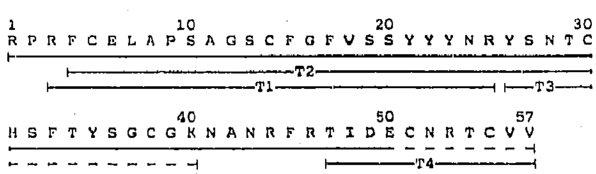

Fig. 2. Primary structure of the Kunitz-type chymotrypsin inhibitor-like polypeptide. Solid lines indicate peptide parts analyzed by sequencer degradations, broken lines remaining parts. T1–T4 constitute the tryptic peptides analyzed.

4. DISCUSSION

The primary structure of this novel inhibitor from *Naja naja naja* venom was determined. Nearly the entire structure was obtained by degradation in a solid-phase instrument for 50 cycles. The remaining part was completed by analysis of the tryptic peptides of the radiolabelled S-carboxymethylated inhibitor. The 57-residue structure has 6 Cys positions. Their pattern and an entire-chain residue homology identify the venom component as a Kunitz-type inhibitor, with the Cys positions corresponding to the 6 invariant half-cystine residues of these inhibitors. Known relationships for the basic pancreatic trypsin inhibitor and other members within this family also allow functional conclusions on protease-interacting residues at critical positions [10].

The residue corresponding to the main contact site (P1) is Phe-15 (Fig. 3). The presence of residues like Phe, Leu and Tyr at this position are supposed to be typical for chymotrypsin inhibitors and we therefore conclude that the new cobra venom component characterized is a chymotrypsin-type of inhibitor. Components with such P1 residues have been observed in the hemolymph of larvae of a silkworm, *Bombyx mori*, having Phe [12], and from the venom of *Vipera ammodytes*, having Leu at this position (Fig. 3). Both are strong inhibitors towards α-chymotrypsin, with dissociation constants K_i of 4.3×10^{-9} M and 1.3×10^{-8} M, respectively, while similar peptides having Tyr at this position have been isolated from the venom of

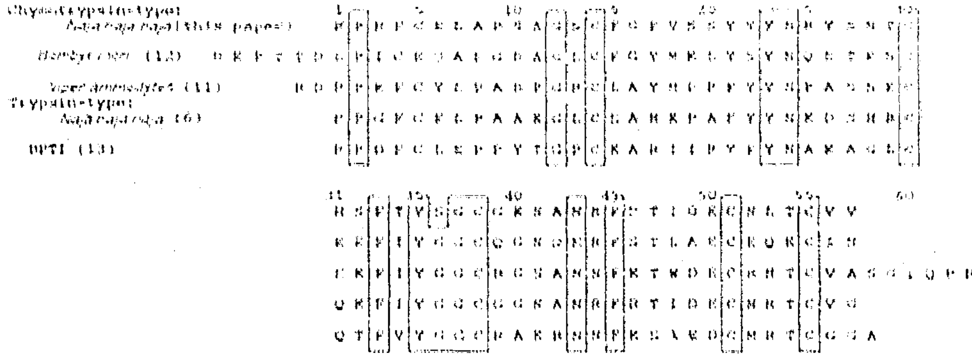

Fig. 3. Alignment of the chymotrypsin inhibitor-like polypeptide now characterized (top line) with the trypsin inhibitor from the same species (line 4), the Kunitz-type chymotrypsin inhibitors previously characterized (species as listed lines 2 and 3) and the model bovine pancreatic trypsin inhibitor (BPTI).

Dendroaspis polylepis and *D. angusticeps*, but with low activity [4,7].

Residues at other positions thought to be involved in protease binding, including those with weak interactions, like Gly-12 (P4), Tyr-23, Asn-24, Phe-33, Tyr-35, Gly-37, Asn-43, and Phe-45 [14] are largely conserved. Only the residue at position 36 is different in the novel *Naja* molecule, being Ser instead of an otherwise conserved Gly-36 (Fig. 3). Consequently, both the trypsin inhibitor recently characterized from *Naja naja naja* [6] and the chymotrypsin-type of inhibitor now detected from the same species, exhibit special properties with deviating residues of functional importance. In the former case, a different residue at P1, probably explaining a strong inhibitory power [6] and in the present case a deviation in an otherwise conserved residue at a weak contact site.

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