# PRIMARY STRUCTURE OF PROTEINASE INHIBITOR II ISOLATED FROM THE VENOM OF RUSSELL'S VIPER (*VIPERA RUSSELLI*)

Hidenobu TAKAHASHI, Sadaaki IWANAGA, Yasuji HOKAMA, Tomoji SUZUKI and Tsunehiro KITAGAWA

*Division of Plasma Proteins, Institute for Protein Research, Osaka University, Suita, Osaka-565, Japan*

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## 1. Introduction

Basic proteinase inhibitors of trypsin and related enzymes are widely distributed in the animal tissues including pancreas, lungs, parotide glands and liver [1]. A number of these have been purified and characterized, including their primary structures [2]. The use of these inhibitors as models for the study of structure–function relationships is particularly attractive. Previously, two novel inhibitors which inhibit kallikrein, plasmin, α-chymotrypsin and trypsin have been found in the venom of Russell's viper, and one of them designated as RVV inhibitor II was characterized to be a polypeptide consisting of 60 amino acid residues [3]. This paper describes the complete amino acid sequence and locations of disulfide bridges in the inhibitor.

## 2. Materials and methods

RVV inhibitor II previously isolated from 900 mg of the venom of Russell's viper (Sigma Chemical Co., St. Louis, lot, S62B-206) was used [3]. The amino acid composition of the inhibitor II determined with 24, 48 and 72 hr hydrolyzates was as follows: $Lys_3$, $His_2$, $Arg_7$, $Asp_8$, $Thr_3$, $Ser_2$, $Glu_5$, $Pro_2$, $Gly_8$, $Ala_2$, ½ $Cys_6$, $Val_1$, $Ile_1$, $Leu_3$, $Tyr_3$ and $Phe_4$. TPCK-trypsin (twice crystallized) and α-chymotrypsin (three times crystallized) were products of Sigma Chemical Co., Thermolysin was a generous gift from Dr. K. Morihara, Shionogi Research Laboratory Co., Osaka. Carboxypeptidase A and B (DFP-treated) were obtained from Worthington Biochemical Corp, Freehold. Sephadex G-25 (superfine) was a product of Pharmacia, Uppsala.

The reduction and carboxymethylation of the inhibitor II (2.9 μmoles) were performed according to the method of Crestfield [4], and the resulting Cm-derivative was gel-filtrated on a Sephadex G-10 column (2.0 × 130 cm) and eluted with 10% acetic acid. Digestions of the Cm-derivative with TPCK-trypsin and α-chymotrypsin were made in 0.2 M $(NH_4)HCO_3$ buffer, pH 8.5, at 37°C, and at 40°C thermolysin digestion with a substrate concentration of 0.5% (w/v) and an enzyme to substrate ratio of 1:50 in all the cases. Some peptide mixtures of the tryptic digest were separated by chromatography on a Dowex 50 (X2) column (0.9 × 40 cm), equilibrated with pyridine acetate buffer, pH 3.05. The peptides were eluted by using a buffer gradient system described by Iwanaga et al. [5]. The peptides were purified also by high-voltage paper (Toyo No. 51A) electrophoreses with pyridine acetate buffer, pH 3.4 and 6.4. Amino acid analysis was performed on samples, which were hydrolyzed at 110° in evacuated sealed tubes with 5.7 N HCl for 24 hr. Evaporated hydrolyzates were analyzed by an amino acid analyzer, Model JLC-5AH, Japan Electron Optics Laboratory Ltd., according to the method of Spackman et al. [6]. Amino acid sequence was analyzed by Edman's PITC procedures [7], and the resulting PTH amino acids were identified by thin-layer chromatography on silica gel plates (Kiesel gel F254), using solvents D and E of Edman and Sjöquist [8] and solvents II and III of Brenner et al. [9].

*Abbreviations*: RVV, Russell's Viper Venom; PITC, Phenyl*iso*-thiocyanate; PTH, Phenylthiohydantoin; Cm, S-carboxyl-methyl; PBTI, Pancreatic basic trypsin inhibitor; CTI, Colostrum trypsin inhibitor; TPCK, L-(1-*p*-Toluenesulfonylamido-2-phenyl)-ethyl chloromethyl ketone.

Table 1
Partial amino acid sequence of tryptic fragments of RVV inhibitor I.

| | |
|---|---|
| RVV inhibitor II | His–Asp–Arg–Pro–Thr–Phe–<br>0.11 0.14 0.04 0.08 0.08 0.05 |
| T2 | His–Asp–Arg–Pro–Thr–Phe–Cm–Asn–Leu–Ala (Glu, Gly, Ser, Pro)–Arg–OH<br>0.26 0.30 0.16 0.18 0.14 0.16 0.12 0.10 0.07 0.05 |
| T-4 | Val–Phe–Phe–Tyr–Gly–Gly–Cm–Gly–Gly–Asn(Ala, Asn, Asn)Phe–Glu–Thr–Arg–OH<br>0.38 0.25 0.20 0.17 0.13 0.08 0.12 0.04 0.02 0.01 0.15 0.06 |
| T-4-4 | Gly–His–Leu–Arg–OH<br>0.23 0.10 0.04 |
| T-5-1 | Gln–Thr–Cm–Gly–Gly–Lys–OH<br>0.06 0.02 0.01 |
| T-5-2 | Asp–Glu–Cm–Arg–OH<br>0.34 0.28 0.18 0.05 |
| T-5-3 | Cm–Lys–OH<br>0.34 |
| T-5-4 | Ile–Tyr–Try–Asn–Leu–Glu–Ser–Asn–Lys–OH<br>0.26 0.25 0.25 0.20 0.16 0.16 0.07 0.03 |
| T-7-2 | Cm–Arg–OH<br>0.14 0.03 |
| T-7-3 | Arg–OH<br>0.19 |

The amino acids cleaved by direct Edman procedure are indicated by arrows (→) under which their molar recovery is given.

## 3. Results

Using native RVV inhibitor II (0.4 μmoles), Edman's stepwise degradation was performed and the partial N-terminal sequence is given in table 1. In each degradation cycle one main PTH-derivative was released together with traces of other PTH amino acids. To determine the complete sequence of the inhibitor II, the Cm-derivative (2.8 μmoles) was digested at 37°C for 6 hr by trypsin and α-chymotrypsin, respectively. In the tryptic digest, peptide mixture was separated first on a Sephadex G-25 column (2.0 × 140 cm) and some of the peptide fractions obtained were then separated by ion exchange chromatography on a Dowex 50 (X2) column. Through these procedures, eight major peptides and free arginine were isolated and their amino acid compositions are shown in table 2. A sum of the total residues of tryptic peptides agreed with whole amino acid composition of the starting material. Table 1 shows also the amino acid sequences of tryptic peptides analyzed by PITC and carboxypeptidase methods [10]. In these peptides, T–2 was deduced to be derived from the N-terminal part of the inhibitor II, as its 6 N-terminal sequences were identical to those of the native material. To obtain overlaps of the tryptic peptides, α-chymotrypsin digest was separated with the same methods as those used on the tryptic digest. Six main peptides and free tyrosine and phenylalanine were isolated and their amino acid compositions and partial sequences were determined. α-Chymotryptic peptides, designated as C–1, C–8–4, C–5–3 and C–4–2 (fig. 1), apparently constituted the overlaps of the tryptic peptides. From these results, all of the alignment of the tryptic peptides could be established.

To determine the location of disulfide bridges and to confirm further the amino acid sequences deduced from the analyses of tryptic and α-chymotryptic peptides, thermolytic digestion on native inhibitor II (1.1 μmoles) was performed at 40°C, pH 6.5, for 12 hr. The digest was first separated by

Table 2
Amino acid compositions of tryptic fragments of RVV inhibitor I.

| Amino acid | T–2 | T–4 | T–4–4 | T–5–1 | T–5–2 | T–5–3 | T–5–4 | T–7–2 | T–7–3 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | residues/mole | | | | | |
| Asp | 2.12 (2) | 3.17 (3) | | | 1.00 (1) | | 2.15 (2) | | |
| Thr | 0.97 (1) | 1.05 (1) | | 1.05 (1) | | | | | |
| Ser | 0.96 (1) | | | | | | 0.98 (1) | | |
| Glu | 1.08 (1) | 1.11 (1) | | 1.13 (1) | 1.05 (1) | | 1.10 (1) | | |
| Pro | 2.00 (2) | | | | | | | | |
| Gly | 1.17 (1) | 4.24 (4) | 1.00 (1) | 2.18 (2) | 0.13 | | | | |
| Ala | 0.94 (1) | 0.96 (1) | | | | | | | |
| Cm | 0.93 (1) | 0.90 (1) | | 0.82 (1) | 0.99 (1) | 0.96 (1) | | 1.00 (1) | |
| Val | | 0.94 (1) | | | | | | | |
| Ile | | | | | | | 1.00 (1) | | |
| Leu | 1.07 (1) | | 1.12 (1) | | | | 1.10 (1) | | |
| Tyr | | 0.94 (1) | | | | | 1.35 (1-2) | | |
| Phe | 1.04 (1) | 2.94 (3) | | | | | | | |
| Lys | | | | 1.01 (1) | | 1.04 (1) | 1.07 (1) | | |
| His | 0.90 (1) | | 0.90 (1) | | | | | | |
| Arg | 1.90 (2) | 1.11 (1) | 0.99 (1) | | 0.96 (1) | | | 1.40 (1) | 1.00 (1) |
| $NH_3$ | 1.90 | 2.7 | 1.26 | 3.14 | 0.89 | 0.84 | 2.96 | 1.24 | 1.39 |
| Total | 15 | 17 | 4 | 6 | 4 | 2 | 8-9 | 2 | 1 |
| Yield (%) | 50 | 29.5 | 36.2 | 14.7 | 44.25 | 39.8 | 28.5 | 37.2 | 27.2 |

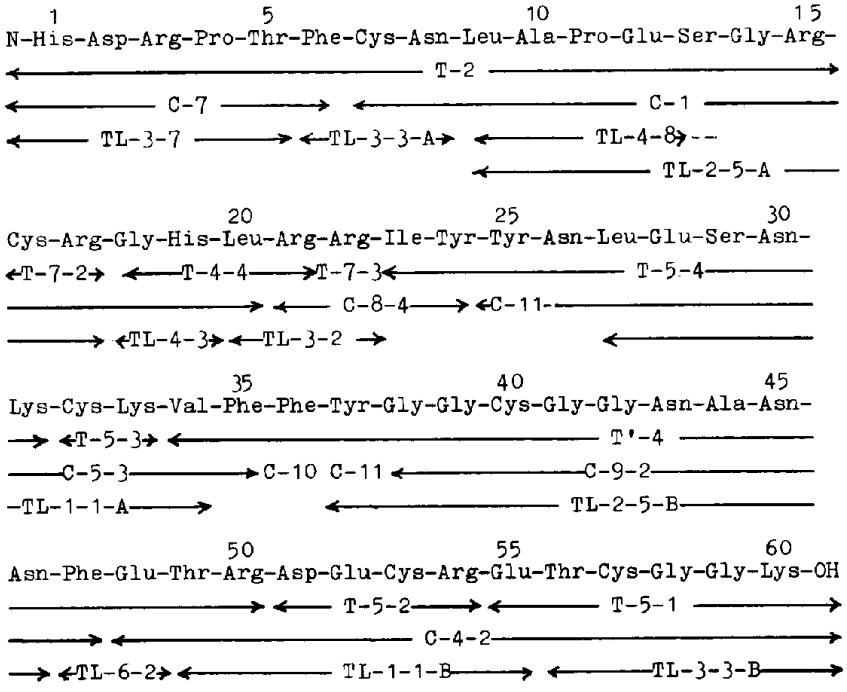


Fig. 1. Amino acid sequence of proteinase inhibitor II from the venom of *Vipera russelli*.

gel filtration on a Sephadex G-25 column (1.5 × 110 cm), and then purified by high-voltage paper electrophoresis at pH 3.4. Three major peptides positive to nitroprusside reaction were isolated and their amino acid compositions were determined. Moreover, on each of the peptides containing disulfide bridges, performic oxidation was made. Two different cysteic acid peptides from each of the cystine peptides were isolated by high-voltage paper electrophoresis at pH 6.4, and their amino acid compositions and partial sequences were determined. These experiments made it possible to elucidate that three disulfide bridges in RVV inhibitor II are located as follows: Cys 7–Cys 57, Cys 16–Cys 40 and Cys 32–Cys 53.

## 4. Discussion

Snake venom contains a number of physiologically active polypeptides including so-called neurotoxins, cardiotoxins and cytotoxins. The chemical structures of these polypeptidic toxins have recently been elucidated [11]. The proteinase inhibitor presented here is quite different substance from above components in its chemical structure and biological activity. Similar proteinase inhibitors have been found also in several venoms of the Elapidae family [12].

As previously reported [3], RVV inhibitor II combines with trypsin in a molar ratio of 1:1 and its inhibitory capacity seems identical with that of pancreatic secretary (Kazal type) and basic (Kunitz type) trypsin inhibitors. The chemical characterizations of RVV inhibitor II indicate that the molecule is very similar to that of bovine PBTI. The N-terminal sequence of RVV inhibitor II was first deduced to be Asp–Asp– by analysis with Edman degradation [7]. However, further investigations on that part revealed the sequence of His–Asp– (fig. 1). The contradiction of these results could be considered as an error due to the abnormal behaviour of histidyl peptides encountered often in the stepwise degradation technique [13]. The overall primary structure of RVV inhibitor II has quite similarity to those of bovine PBTI [14, 15] and cow's colostrum trypsin inhibitor (CTI) [16]. The 6 half-cystine of three inhibitors occupy the same positions in the amino acid sequences of these polypeptides. Moreover, about 50% homology of the amino acid sequences between RVV inhibitor II and PBTI could be found, when chemical similarities are scored on the basis of Lys:Arg, Asp:Glu Asp:Asn, Glu:Gln Thr:Ser, Val:Ile, Ile:Leu and Tyr:Phe [17]. The reactive site of RVV inhibitor II is still unknown, but most probably site, which interacts with trypsin, would be a peptide linkage of Arg 17–Gly 18, because the sequence homologies around this portion between two inhibitors are extremely high. During the course of this study, Strydom [18] reported that a large part of the amino acid sequences of toxins I and K, which were isolated from the venom of *Dendroaspis polylepis* (black Mamba), are very similar to those of PBTI and CTI. Differing from PBTI and CTI, those toxins were reported as monovalent type proteinase inhibitor, since toxin K inhibits only trypsin but not α-chymotrypsin, whereas the reverse is true on toxin I. Thus, RVV inhibitor II, which is a polyvalent rype inhibitor, seems to be much similar to PBTI and CTI. Their structural similarities also support this idea.

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