ISOLATION OF A NOVEL INHIBITOR OF KALLIKREIN, PLASMIN AND TRYPSIN FROM THE VENOM OF RUSSELL'S VIPER (*VIPERA RUSSELLI*)

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1. Introduction

Hageman factor (Factor XII) is found in mammalian blood plasma and it activates plasma prekallikrein directly [1–2]. It is known that snake venoms contain protein components related to kallikrein–kinin and blood-coagulation systems, so we looked for a prekallikrein activator in snake venoms. Unexpectedly, no prekallikrein activating activity was found in snake venoms, but during these studies we found a potent kallikrein inhibitor in the venom of Russell's viper. This factor also strongly inhibited the activities of plasmin and trypsin. It was found to be a polypeptide with a molecular weight of about 7,000 and amino terminal aspartic acid. This paper reports the isolation of this proteinase inhibitor from the venom and its characterization.

2. Materials and methods

The venom of *Vipera russelli* (lot S62B-206) was obtained from Sigma Chemical Co. Bovine trypsin (three times crystallized) was from Worthington Biochemicals Corp. α-Bungarotoxin was a preparation made by Dr. D. Mebs, Frankfurt (Germany) [3]. Purified bovine plasma kallikrein [4] and bovine plasmin [5] were prepared by the methods reported previously. Commercial hog pancreatic kallikrein was further purified as reported previously [6]. Sephadex G-75, G-50 and SE-Sephadex C-25 (2.3 meq/g) were purchased from Pharmacia, Uppsala, Sweden. Polyacrylamide gel electrophoresis was carried out by the method of Davis [7] in a disc electrophoresis apparatus, analytical model (M and S Kiki Ltd., Japan) at pH 4.0 using 7.5% gels. The gels were stained with Coomassie brilliant blue R250 (Colab Labs., Chicago Heights, USA). Molecular weights were determined using SDS-polyacrylamide gel disc electrophoresis by the method of Shapiro et al [8]. Whale sperm myoglobin, bovine chymotrypsinogen-A, ovalbumin and horse heart cytochrome *c* from Schwarz-Mann, USA were used as marker proteins. The amino terminal residue was identified by following Edman's phenylthiohydantoin procedure [9] and identifying the resulting PTH amino acids by thin-layer chromatography on silica gel plates (Kieselgel F_{254}, Merck, Darmstadt, Germany). The activities of kallikreins, plasmin and trypsin were measured at 37° using TAME as substrate. For determination of inhibitory activity, a mixture of enzyme and inhibitor in 0.9 ml of 0.4 M Tris-HCl buffer, pH 8.5, was incubated for 20 min, and then 0.1 ml of 0.1 M TAME was added. After 30 min, the amount of TAME hydrolyzed was determined by the hydroxamate method of Roberts [10]. One inhibitory unit is defined as the amount causing reduction of TAME hydrolysis by 1 μmole per min.

* *Abbrevations:* RVV, Russell's Viper Venom; TAME, *N*-α-tosyl-L-arginine methylester; SDS, sodium dodecylsulfate.

3. Results

3.1. *Isolation of RVV inhibitors*

Lyophilized Russell's viper venom (100 mg) was dissolved in 2 ml of elution buffer and applied to a Sephadex G-75 column (2.5 × 128 cm), equilibrated

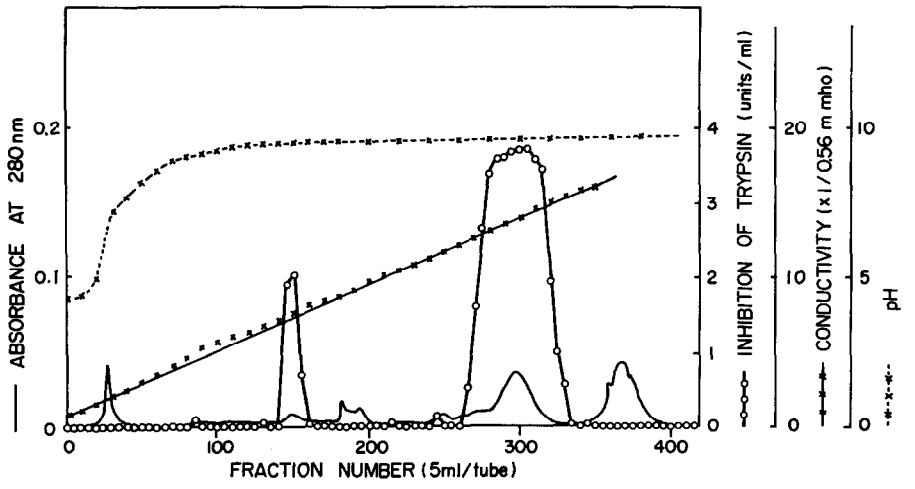

Fig. 1. Purification of the RVV inhibitor on SE-Sephadex C-25. The fraction (15 mg) with inhibitory activity from a Sephadex G-75 column was applied to a SE-Sephadex column (2.0 × 30 cm), equilibrated with 0.04 M ammonium formate buffer, pH 4.0. Linear gradient elution was started with 500 ml of equilibration buffer in the mixing vessel and 0.2 M ammonium acetate buffer, pH 9.5, in the reservoir. Then a gradient of 0.2 M to 0.5 M buffer, pH 9.5 (500 ml each), was used to elute all adsorbed material. Fractions of 5 ml were collected at 4° at a flow rate of 60 ml per hr.

with 0.04 M Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl. Elution was performed with the buffer used for equilibration, and 5 ml fractions were collected at 4° at a flow rate of 50 ml per hr. Aliquots of each fraction were incubated with trypsin (4 μg) for 20 min and then 0.1 ml of 0.1 M TAME was added. After 10 min, the amount of TAME hydrolyzed was determined [10].

Fractions with inhibitory activity were collected and lyophilized, and the buffer used as eluate was removed by passage through a column (2.5 × 94 cm) of Sephadex G-50, equilibrated with 0.1 M NH_4HCO_3 solution. Fractions in the protein peak having inhibitory activity were pooled and lyophilized. The material obtained (15 mg) was applied to a column of SE-Sephadex C-25 (2.0 × 30 cm), equilibrated with 0.04 M ammonium formate buffer, pH 4.0. Linear gradient elution was started with 500 ml of the equilibration buffer in the mixing vessel and 0.2 M ammonium acetate buffer, pH 9.5, in the reservoir. Then the column was eluted with a gradient, formed with 0.2 M buffer to 0.5 M, pH 9.5 (500 ml each), to elute all the adsorbed materials. Fractions of 5 ml were collected at 4° at a flow rate of 60 ml per hr and their inhibitory activity was measured as described above. The inhibitory activity was found in two peaks (fig. 1), and the main peak (tubes no. 280–330) was collected and lyophilized. Ammonium acetate was removed from the dried material in vacuo (0.005 mm Hg) at 40°. The overall yield of the inhibitor (RVV inhibitor) was about 5 mg from 100 mg of venom.

3.2. *Purity and properties of the RVV inhibitor*

As shown in fig. 2, the purified inhibitor gave a single homogeneous band on polyacrylamide gel electrophoresis at pH 4.0 and on SDS-polyacrylamide gel electrophoresis at pH 7.2. Its mobility on the gel was almost the same as that of α-bungarotoxin which has a molecular weight of 7,983 [8]. From these results the inhibitor seemed to be basic protein with a molecular weight of about 7,000 to 8,000. Two cycles of Edman degradation established the N-terminal sequence as Asp–Asp–. The composition of amino acid residues, determined with 24, 48, 72 hr hydrolysates was as follows: Lys_3, His_2, Arg_7, Asp_8, Thr_3, Ser_2, Glu_5, Pro_2, Gly_8, Ala_3, $1/2Cys_6$, Val_1, Ile_1, Leu_3, Tyr_2 and Phe_4. No tryptophan was detected spectrophotometrically [11]. Thus, the material was deduced to have a total of 60 amino acid residues.

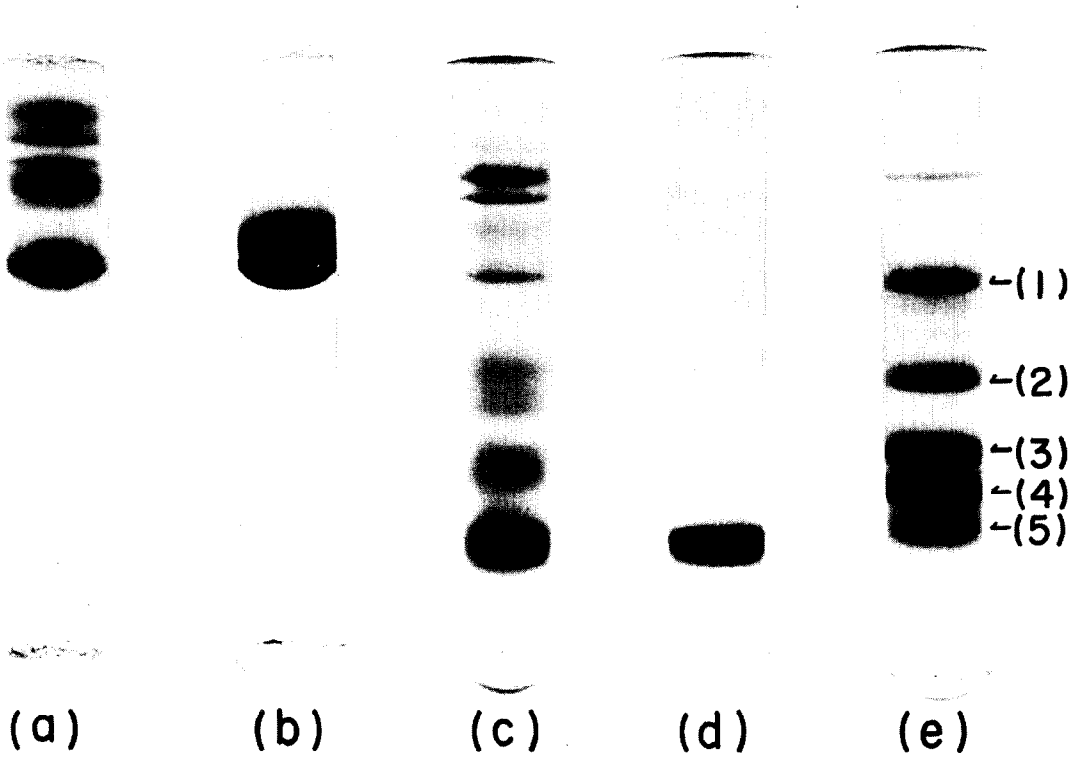

Fig. 2. Disc gel electrophoreses of the RVV inhibitor and unfractionated venom in the absence or presence of SDS. Electrophoresis was done in 7.5% polyacrylamide gel for 1.2 to 4 hr using a current of 4–6 mA per tube. (a) Russell's viper venom (30 μg) and (b) RVV inhibitor (20 μg) at pH 4.5. (c) Russell's viper venom (30 μg), (d) RVV inhibitor (5 μg) and (e) marker proteins in SDS-gel at pH 7.2. (1) Ovalbumin, (2) Chymotrypsinogen-A, (3) Myoglobin, (4) Cytochrome *c*, (5) α-Bungarotoxin.

3.3. *Inhibitory effects of the RVV inhibitor on the activities of kallikreins, plasmin and trypsin*

The RVV inhibitor inactivated plasma and pancreatic kallikreins, plasmin and trypsin and its action on trypsin was quite strong. A titration curve for inactivation of trypsin showed that 1.1 μg of RVV inhibitor completely inactivated the TAME hydrolytic activity of 4 μg of trypsin in 20 min at 37°. So, assuming that one mole of enzyme reacted with one molecule of inhibitor to form an enzyme–inhibitor complex, the molecular weight of the RVV inhibitor was calculated as 6,900. This value is in good agreement with the molecular weight of the inhibitor estimated by results of SDS-gel electrophoresis and amino acid analysis.

4. Discussion

Recently, a number of proteinase inhibitors have been isolated from various natural sources including animal organs, biological fluids and plants [12]. However, proteinase inhibitors have not previously been found in secretory fluid from venomous snakes. This paper reports the isolation of a potent inhibitor of kallikrein, plasmin and trypsin from the venom of Russell's viper, a member of the Viperidae. The inhibitor inactivated trypsin probably by formation of an enzyme–inhibitor complex in a molar ratio of 1:1. Inhibitory activity similar to that of the RVV inhibitor was also found in the venoms of several members of

the Elapidae. The distribution and characterization of these inhibitors will be reported elsewhere [13].

Like the Kazal-type inhibitor and the inhibitor in seminal plasma, the RVV inhibitor was found in a secretory fluid. All these inhibitors contain 56 to 61 amino acid residues per molecule and have no methionine or tryptophan [12]. However, the amino acid composition and N-terminal sequence of the RVV inhibitor, Asp–Asp–, are different with those of the inhibitors so far isolated from mammalian sexual and pancreatic glands. Thus, in studies on the structure of the regions of the inhibitor molecule essential for inhibitor activity, the amino acid sequence of the RVV inhibitor should be determined and compared with the structures of the other inhibitors in secretory fluids. Studies on the amino acid sequence and the inhibition spectrum of the RVV inhibitor are now in progress.

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