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PROTEOLYTIC PROFILE OF *VIPERA AMMODYTES* SNAKE VENOM

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Vipera ammodytes (adder) snake venom is a very complex mixture of numerous substances with a broad spectrum of biochemical and physiological activities. It was separated into nine protein fractions using a Sephadex G-100 column. Molecular masses of the fractions were in the range from just below 10 to 138 kD. General proteolytic activities of venom and its fractions were very similar but the minimal proteolytic dose (MPD) of fractions and whole venom were different. Snake venom contained serine protease inhibitors and serine-dependent proteases were predominant in general proteolytic activity (GPA). Their activities were successfully inhibited by heparin and prostacyclin. Whole snake venom and fractions contained lytic activity against bacteria and fungi. Metalloproteases were predominant in bacteriolytic activity.

Key words: snake venom, proteases, inhibition

INTRODUCTION

A prominent feature of viper venoms is an abundance of proteolytic enzymes. The venom contains several serine proteases and a variety of metalloproteases whose biological effects are poorly understood. Among metalloproteases, the hemorrhagins play an important role in the envenomation. Those substances show different biochemical and physiological activities. The results are local tissue damage, hemorrhage, oedema, myonecrosis and fibrinogenolysis, fibrinolysis etc (Labib et al., 1981) . Poisoning is a result of the simultaneous action of toxins and proteolytic enzymes present in snake venom.

The venom of the *Viperidae* family usually contains at least two types of proteolytic enzymes: heat stable endopeptidases which are inhibited by DFP and usually related to thrombin-like enzymes and kininogenases and heat labile proteins, active on casein or hemoglobin and inhibited by metal chelating agents (Iwanaga et al., 1979). *Vipera ammodytes* (adder) belongs to the most poisonous and clinically important snakes in Europe and its toxin exhibits permanent cardiotoxic, neurotoxic and cytotoxic effects. Different substances have been tested for their ability to prevent the toxic and proteolytic activity of snake venom: specific antisera (Petković et al., 1991), basic and acidic organic compounds (Zdjelar et al., 1990, Cvetković et al., 1990), imunoglobulins (Catanese et al.,

1985), inorganic and organic calcium (Petković et al., 1988). None of these substances could completely inhibit the activity of snake venom. Therefore, investigation of the nature and mechanism of action and inhibition of proteolytic enzymes and toxins from *Vipera ammodytes* snake venom is of considerable importance because neither toxic nor proteolytic activities have been appropriately characterised. Since proteolysis is a very important process in the whole living world, we studied the proteolytic activity of snake venom of *Vipera ammodytes*, using different substrates and inhibitors. Here, we also investigated the ability of some substances suitable for medical trial to prevent the proteolytic activity of snake venom.

MATERIAL AND METHODS

Separation of Vipera ammodytes venom. Dried venom of *Vipera ammodytes* (Sigma, USA) was separated by means of gel-chromatography, using a Sephadex G-100 column, prepared in 0.01 mol/L Tris-HCl buffer (pH 7.5). Crude venom (150 mg) was dissolved in 1 mL of the same buffer and applied to the column. Elution was continued with 0.01 mol/L Tris-HCl buffer and 4 mL fractions were collected and analysed at 280 nm. Pooled fractions were lyophilized, dissolved in 2 mL of saline solution and dialysed against the same solution.

Molecular mass estimation. The molecular masses of snake venom fractions were determined using the same column. Marker proteins used for the calibration were: alcohol-dehydrogenase (148 kD), bovine serum albumin (67 kD), creatine-kinase (81 kD), peroxidase (40 kD) and cytochrome c (12,4 kD).

Protein content determination. Protein content in whole venom and its fractions was determined by the Bradford method (Bradford , 1976).

Proteolytic activity. The proteolytic activity was determined as general proteolytic activity using casein or human serum albumin (Hashimoto et al., 1963). One unit of the general proteolytic activity on casein (GPA-C) was expressed also as minimal proteolytic dose (MPD), i.e. the amount of enzyme which causes a change in absorbance at 280 nm of 0.5 (Gutierrez et al., 1995) . One unit of the general proteolytic activity on human serum albumin was expressed in Pronase Unit Kaken (PUK) - the amount of enzyme which causes an increase of absorbance at 660nm by 1.0 under the test conditions.

Aminopeptidase activity. The synthetic substance L-leucine-4-nitroanilide was used as the substrate, according to the method of Pfleiderer et al.(1964). One unit (U) of enzyme activity is the amount of enzyme which catalyses the transformation of 1 μmol of substrate in one minute.

Lytic activity. Heat - killed cells of Gram negative bacteria *Escherichia coli*, ATCC 25922, Gram positive bacteria *Staphylococcus aureus* ATCC 25923 and yeast-like *Candida albicans* ATCC 24433 were used as substrates. Lytic activity was examined by the method of Stepanov et al.(1980). One unit of lytic activity is the amount of enzyme which decreases the absorbance at 540 nm by 1 % in one minute.

Antimicrobial activity. The antimicrobial activity of the whole venom was examined by an antibiogram test (Vanden Bergie et al., 1991). The test microorganisms used were: *E. coli, C. albicans* and *S. aureus*.

Inhibition tests. Specific inhibitors of functional groups: EDTA, PMSF and PHMB in 1 mM concentration were investigated for their influence against the proteolytic activity of whole snake venom and its fractions on casein, as well as, against the lytic activity of snake venom. Also, the effect of the following

substances on the general proteolytic activity of whole snake venom on casein was tested: specific antiserum - antiviperin (Torlak, diluted 10x), heparin (Galenika, 5000 IJ) and prostacyclin (Flolan, Welcome, UK, 1,66 μmol/L) in volumes of 0,5 mL. The remaining proteolytic activity was expressed in relation to the control (100 %) activity of the venom without an inhibitor .

Autogenic inhibition. The presence of autogenic inhibitors in *Vipera ammodytes* snake venom was measured as a decrease of the GPA of trypsin against casein and human serum albumin (HSA) after preincubation of the enzyme and snake venom for 15 minutes.

Statistical analysis . Statistical analysis was performed using Students t-test for paired observations. The means ± SEM of n observations were quoted in the text and figures and $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Separation and proteolytic activity of snake venom and its fractions. The separation curve of snake venom is presented in Figure 1.

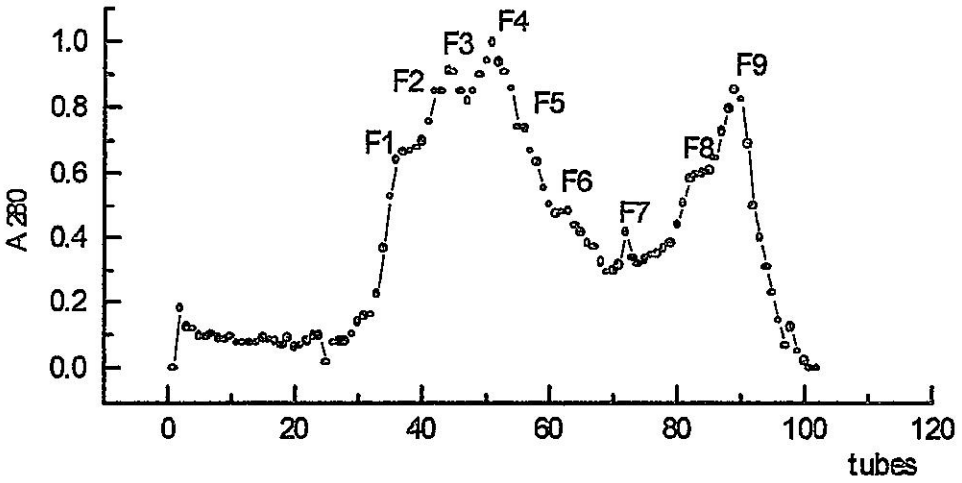

Figure 1. Separation curve of *Vipera ammodytes* snake venom. 150 mg of crude venom was separated on a Sephadex G-100 column, bed dimension 90x2.4 cm. The column was equilibrated with 0.01 mol/L Tris-HCl buffer (pH 7.5). Elution was continued with 0.01 mol/L Tris-HCl buffer and 4 mL fractions collected at flow rates of 24 mL/h. F1-9- peaks of fractions.

Gel-chromatography on Sephadex G-100 gave 9 fractions with a yield of 53 %. It was reported that under the same conditions 7 (Muić et al., 1995) or 11 (Mićević, 1989) fractions could be separated. The number of protein fractions depended on factors such as: season of venom collecting, storage, temperature, light etc. These factors could decrease proteolytic activity of snake venom but they did not influence toxicity.

Molecular masses (MM) of fractions, their contribution (C) in the whole venom, specific general proteolytic activity toward casein (GPA) and minimal proteolytic dose (MPD) are presented in Table 1.

Table 1. Some characteristics of *Vipera ammodytes* venom and its fractions. Molecular masses of fractions (MM) were determined using Sephadex G-100 column chromatography, in the presence of the marker proteins: alcohol-dehydrogenase (148 kD), bovine serum albumin (67 kD), creatine-kinase (81 kD), peroxidase (40 kD) and cytochrome c(12,4 kD). The contribution (C) of separated fractions in whole venom expressed as a %. General proteolytic activity of whole snake venom and its fractions was determined on casein and the activity calculated in Pronase unit Kaken (PUK) per mg of protein. Minimal proteolytic dose (MPD) of whole snake venom and fractions represents the amount of enzyme which causes changes in absorbance at 280 nm by 0.5.

Fraction	MM (kD)	C (%)	GPA (PUK/mg)	MPD (mg)
S*	/	/	0.56	0.373
F1	138.8 +/-7.5	11.4	0.70	0.070
F2	123.8 +/-4.5	12.7	0.44	0.109
F3	114 +/-4	8.4	0.45	0.110
F4	78.8 +/-6.5	34.5	0.48	0.104
F5	58.5 +/-6	16.8	0.55	0.087
F6	43.5 +/-4.5	8.2	0.77	0.064
F7	21.3 +/-4	7.1	1.14	0.043
F8	<10	0.8	17	0.003
F9	<10	0.2	33.75	0.001

* - whole snake venom

Molecular masses of the fractions F1-F7 were in the range of 138.8-21.3 kD. Fractions F8 and F9 were polypeptides with molecular masses under 10 kD.

The venoms from *V.ammodytes* showed a different arrangement of molecular masses, for example, from the venom produced by Brezje Immunological Institute, Zagreb, which contained 11 protein fractions. The distribution of molecular masses was also significantly different; for instance fractions F3 - F6 had masses from 44 to 10 kD; F7 - F11 had molecular masses between 8 and 5 kD. In the snake venom of *Vipera ammodytes* analysed in our laboratory higher molecular mass fractions F1 to F7 were detected although two fractions F8 and F9 also contained peptides with molecular masses under 10 kD.

The general proteolytic activity was determined as caseinolytic and in Table 1 it could be seen that the values for whole snake venom and its fractions were in the range 0.44 to 1.14 PUK/mg except for fractions F8 and F9 which had, 17 and 33.75 PUK/mg, respectively. We also expressed GPA as a minimal proteolytic dose. The values for whole snake venom corresponded well to those given in the literature. Thus for some *Bothrops* species they were as follows(mg): *B. asper* -1.2; *B. nummifer* - 0.5; *B. godmani* - 0.6. The MPD was 0.9 mg for *Crotalus durissus durissus* and 0.9 mg for *Lachesis muta* (Kress et al., 1981). The MPD values for

snake venom fractions were not given. It has to be underlined that we have detected fractions with extreme MPD (F5-F9) and those fractions had significantly different MPD in comparison to whole snake venom.

It was very interesting that the *Vipera ammodytes* venom and its fractions were without Leu-aminopeptidase activity.

Inhibition of general proteolytic activity (GPA) of trypsin by whole snake venom. Trypsin showed lower activity in the presence of *V.ammodytes* snake venom proteases. The inhibition was 26,7 % and 36,7 % towards casein and human serum albumin, respectively, and it was evident that *V. ammodytes* snake venom was the source of serine protease inhibitors. Serine protease inhibitors were found earlier in *V.ammodytes* venom (Ritonja et al., 1981).

Inhibition of proteolytic activity of Vipera ammodytes snake venom. The general proteolytic activity of *Vipera ammodytes* snake venom and its fractions was not significantly inhibited by 1 mM EDTA, and therefore we concluded that the source of general proteolytic activity was not metalloproteases. Similar experiments showed that the GPA of *Cerastes cerastes and Cerastes vipera* whole venom proteases was completely inhibited by 1 mM EDTA; thus C. cerastes and C. vipera contained predominantly metalloproteases (Labib et al., 1981).

On the other hand, general proteolytic activity of snake venom of *Vipera ammodytes* was completely inhibited in the presence of PMSF: (Sigma -100 %, Torlak - 92 %) and PHMB (Sigma -83 %, Torlak - 98 %). This indicated that serine and thiol - dependent proteases dominated in the GPA of the snake venom. If autogenous serine protease inhibitors existed, serine protease would be expected to be an important part of the proteolytic activity.

Recently, the inhibition of serine proteases by some substances suitable for clinical trial was reported (Cvetković et al., 1988). Therefore, heparin and prostacyclin were tested for inhibition ability against snake venom proteases. The activity of these substances was compared with antiviperin as an antitoxic substance which was administered in the envenomation (Fig. 2)

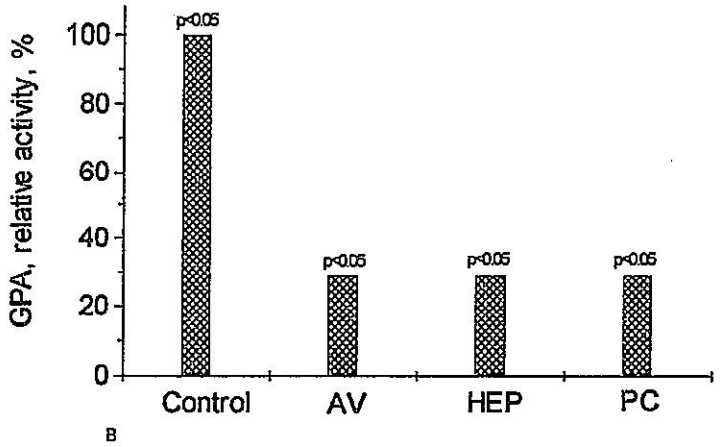

Figure 2. Effect of antiviperin (AV), heparin (HEP) and prostacyclin (PC) on general proteolytic activity of *Vipera ammodytes* snake venom. General proteolytic activity examined on casein solution (1 %) in 0.1 mol/L Tris HCl buffer (pH 8.0). In the presence of AV (0.5 mL of the ten times diluted commercial Torlak preparation) GPA was reduced to 30 %. Heparin (0.5 mL , concentration 5000 IU/mL) decreased GPA to 30 %. In the presence of prostacyclin (0.5 mL, concentration 1.66 çmol/L) 30 % of GPA remained.

Heparin and prostacyclin were very efficient inhibitors of GPA (71 %), similarly to antiviperin (also, 71 %). The efficiency of heparin and prostacyclin could be due to the inhibition of serine proteases present in *V. ammodytes* snake venom (Petković et al., 1991).

Lytic activity of Vipera ammodytes snake venom and inhibition of lytic activity.

Lytic activity in different proteolytic mixtures was detected. Snake venom of *Vipera ammodytes* as a natural mixture with significant proteolytic activity is a potential source of lytic activity, but no evidence was found in the literature. The antibacterial activity of 30 chosen species from the *Elapidae* and *Viperidae* families active against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherishia coli*, *Aeromonas hydrophila* and *Bacillus subtilis* was investigated. In all cases antimicrobial activities were small (Stiles et al., 1991). Our investigation of the antimicrobial activity of *V. ammodytes* venom by the same technique did not show any antimicrobial action towards Gram positive or Gram negative bacteria or *C. albicans*, but significant lytic activity of *V. ammodytes* was obtained as shown in Table 2.

Table 2. Lytic activity *of Vipera ammodytes* snake venom. Lytic activity of the whole venom and its fractions was examined on the test-microorganisms E.coli (Gram negative bacteria), S.aureus (Gram positive bacteria) and C.albicans (yeast-like), and expressed in U of lytic activity. One U of lytic activity is the amount of enzyme which decreases the absorbance at 540 nm by 1 % in 1 minute.

Fraction	*E.coli* (U)	*S.aureus* (U)	*C.albicans* (U)
S*	6.6	3.2	0
F1	19.6	5.2	7.4
F2	14.0	0	15.3
F3	19.6	6.0	0
F4	16.6	3.8	9.0
F5	20.0	0	0
F6	0	6.9	8.6
F7	12.0	0.7	0
F8	18.0	3.8	0
F9	27.6	8.0	4.4

* - whole snake venom

Bacteriolytic activity of the *Vipera ammodytes* venom and its fractions, determined as the ability to lyse heat-killed *E. coli* cells, was present in all fractions except F6. Maximal bacteriolytic activity was in fraction F9 - 27.6 U. Lytic activity towards heat-killed *S. aureus* cells was low and it was absent in fractions F2 and F5. Fractions F3, F5, F7 and F8 were not active against heat-killed cells of *C. albicans*. From these results it could be concluded that all fractions except F5 were able to lyse heat-killed cells of the three test microorganisms used; only fraction F5 contained bacteriospecific lytic activity. The other fractions contained enzymes that hydrolyzed microorganism cells by nonspecific proteolytic activity.

Thus, *V. ammodytes* snake venom was not found to be antimicrobial but possessed significant bacteriolytic activity.

The influence of EDTA, PMSF and PHMB on bacteriolytic activity of whole snake venom and four characteristic fractions is presented in Figure 3. In particular, F1 was active toward alls microorganisms tested, while F3 was found to be active toward G+ and G- bacteria. The fraction F7 showed activity towards G+ bacteria while F9 contained nonspecific low molecular lytic activity and was active against all microorganisms.

Bacteriolytic activity of venom and fractions was completely inhibited in the presence of EDTA. Thus, bacteriolytic enzymes were metalloproteases. The lytic activity was activated in the presence of PMSF and PHMB. It seemed that the activity of lytic enzymes was controlled by serine/thiol proteases present in fractions of *V. ammodytes* snake venom.

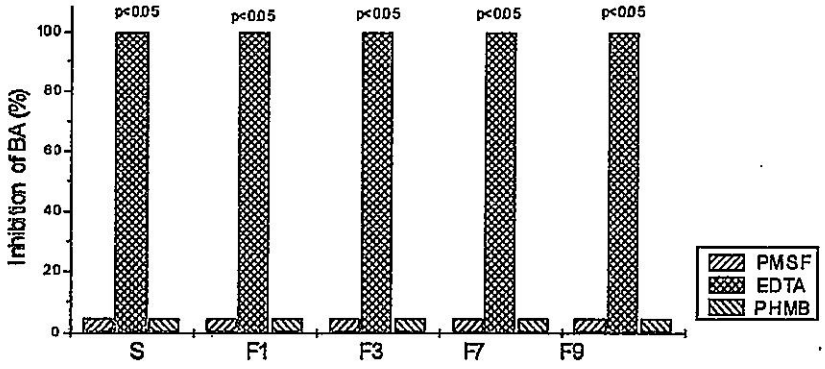

Figure 3. Influence of EDTA, PMSF and PHMB on bacteriolytic activity (BA) of *V. ammodytes* snake venom. Ethylene-diamine-tetraacetic acid (EDTA) in a concentration of 1 mmol/L inhibited BA (examined on heat-killed *E.coli* cells) of whole venom (S), high molecular weight fractions F1 and F3 and low molecular weight fractions F7 and F9 by 100 %. On the contrary, in the presence of 1 mmol/L phenylmethylsulfonyl-fluoride and p-hydroxymercury-benzoate whole snake venom and all examined fractions were completely active.

Conclusion

Snake venom of *Vipera ammodytes* was separated into nine protein fractions, with molecular masses from 138.8 to under 10 kD. The GPA of the whole snake venom and fractions 1 to 9 were similar, but MPD was different. In general proteolytic activity of snake venom was predominantly due to serine and thiol-dependent proteases, since its activity was completely inhibited by PMSF and PHMB. General proteolytic activity of the whole venom could be inhibited by heparin and prostacyclin by approximately 70 % which is similar to the commercial antitoxic drug antiviperin.

Bacteriolytic activity of snake venom and its fractions determined as the ability to lyse heat-killed *E. coli* cells, was present in the whole snake venom and in all fractions except F6. Also, since all fractions were able to lyse heat killed cells of the three test microorganisms except F5; we presumed that only fraction F5

contained bacteriospecific lytic activity. *V. ammodytes* snake venom did not have antimicrobial activity.

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PROTEOLITIČKI PROFIL OTROVA ZMIJE *VIPERA AMMODYTES*

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SADRŽAJ

Otrov zmije *Vipera ammodytes* je složena smeša brojnih supstanci sa širokim spektrom biohemijskih i fizioloških aktivnosti. Primenom kolone Sephadex G-100 ova smeša je razdvojena u 9 frakcija. Molekulske mase frakcija bile su od 138 do ispod 10 kD. Vrednosti opšte proteolitičke aktivnosti otrova i frakcija su vrlo slične, ali se MPD vrednosti frakcija razlikuju; otrov pokazuje maksimalnu vrednost za MPD, dok su ove vrednosti za frakcije različite. Ukupni zmijski otrov sadrži inhibitore serin-proteaza i u OPA su dominantne serin zavisne proteaze; njihova aktivnost se može efikasno inhibirati primenom heparina i prostaciklina. Ukupni zmijski otrov i frakcije pokazuju fungilitičku i bakteriolitičku aktivnost. Metaloproteaze preovlađuju u litičkoj aktivnosti.