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AGE CHANGES OF *VIPERA BERUS* VENOM AMIDOLYTIC ACTIVITY

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A. A. NEDOSPASOV and E. V. RODINA. Age changes of *Vipera berus* venom amidolytic activity. *Toxicon* **30**, 1505–1508, 1992.—The study of age changes of amidolytic activity of *Vipera berus* venom is investigated using mixtures of chromogenic peptide substrates differing by detected groups. Quantities of venom (total protein content) and its proteolytic activity from snakes of different ages were compared. The venom composition of newly born adders was shown to be considerably different from the venom composition of young (12-month) adders of the same population. The next evolution of protease activity is weakly expressed.

IN THE popular medicine of the Russian North a method of lumbago treatment consists of a newborn adder biting the patient's waist. A bite by a grown adder may be lethal. It remained unclear whether the curative properties of the bite of a young adder were due to the small amount of venom injected or to a difference in composition of the venoms of young and old snakes. Age-dependent variation in the composition of snake venoms is well known (BONILLA *et al.*, 1973; FIERO *et al.*, 1972; MEIER and FREYVOGEL, 1980), and experiments on the splitting of chromogenic peptide substrates play an important role in analysis of age changes of venom proteases activity (MEIER, 1986). In this communication we propose a new approach for these problems. In the ANSA-analysis method (NEDOSPASOV *et al.*, 1989*a*; NEDOSPASOV and CHERKASOV, 1992*a*,*b*) mixtures of chromogenic substrates with aminonaphthalenesulfonamide (ANSA*) chromophore are used and the substrates differ not only in peptide sequences but also by detected groups. The ANSA-spectrum is a chromatographically determined quantitative composition of this group, cleaved of the substrate mixture by the enzymatic preparation under study.

To test the hypothesis of non-identical composition of the venom of young and old vipers, the dependence of the venom protease ANSA-spectrum on the age of vipers was studied. Several ANSA-substrates split by adder venom proteases were revealed in preliminary experiments. Freshly collected venom from animal groups of both sexes was dissolved to standard protein concentration. If the venom composition were invariable the ANSA-spectra of venom samples from young and old adders should be identical.

* *Abbreviations*:—ANSA, aminonaphthalenesulfonamide; Bz, benzoyl; HPLC, high performance liquid chromatography; MCA, 4-methylcoumarine-7-amide; Tos, toluensulfonyl.

TABLE 1. COMPOSITION OF SUBSTRATE MIXTURES

Substrate*	Mixture 1	Mixture 2
Met–ANSA-ethyl		+
Boc–Glu(*O-tert*-butyl)–Gly–Arg-ANSA-ethyl	+	
Arg–ANSA-(3-oxapentamethylene)	+	
Bz-Ile–Glu–Gly–Arg–ANSA-(3-oxapentamethylene)		+
Gly–Pro–ANSA-butyl	+	+
Pro–Phe–Arg–ANSA-diethyl (II)	+	+
Tos–Gly–Pro–Arg–ANSA-(pentamethylene) (I)	+	

*ANSA-substrates were obtained by multi-step synthesis, with ANSA as the C-protected group (NEDOSPASOV *et al.*, 1989*b*). ANSA were either produced by a previously described method (NEDOSPASOV, 1988, NEDOSPASOV and SHARINA, 1992) or obtained from the Institute of Biochemistry, Vilnius, Lithuania.

Peptidase activity was determined in two mixtures of ANSA-substrates, containing 0.54 mM of each substrate and 0.07 mM dansylmorpholine as inner standard in 0.066 M Tris–HCl buffer, pH 8.2. Substrate mixture (0.3 ml) was incubated for 10 min in a plastic Eppendorf tube, with 20 μl of venom; 0.4 ml of ethyl acetate-hexane-acetic acid (200:100:1) mixture was added, the tube was stirred, the upper phase was withdrawn, vacuum-dried and analysed by HPLC. All experiments were done in duplicate. The HPLC analysis of samples was performed on a Du Pont chromatographer with the absorption detection of $\lambda = 340$ nm. Samples were dissolved in 200 μl of 30% CH_3CN, then 50 μl of dissolved samples were separated on 4.6 × 250 mm Zorbax-C-8 column at 35°C using a linear gradient of 50–80% of solution B in solution A during 18 min, where A is 50 mM ammonium phosphate, pH 3; and B is 60% CH_3CN + 40% A. The flow rate was 1.5 ml/min. Peak identification was performed by comparing with standard ANSA samples. The amount of a given ANSA in a sample was determined by the ratio of the ANSA and dansylmorpholine peak squares.

Essential differences of ANSA-spectra were shown experimentally. Substrates used were split far more slowly by young adders' venom and quantitative measurements could only be performed with the two most active substrates: Tos–Gly–Pro–Arg–ANSA (I) (specific for thrombin-like proteases) and Pro–Phe–Arg–ANSA (II) (specific for kallikrein-like proteases). Two substrate mixtures were used in this work (Table 1). Since all substrates present in the mixture at a time may compete for the active centres of venom proteases (all other substrates can be regarded as competitive inhibitors with respect to a given substrate), the cleavage rate of the same substrate will differ depending on the particular substrate mixture, both in the case when several enzymes can cleave the substrate, and in the case of competition of several substrates for the same enzyme. Data of SAMEL *et al.* (1987) show that at least two enzymes splitting D-Pro–Phe–Arg–MCA are present in viper venom, and K_M of the reactions differ by more than an order of magnitude. Substrate II that we used in both mixtures is a close structural analogue of the above peptide, and therefore we have every reason to believe that its splitting by the same proteases was possible. Since the Pro–Phe–Arg sequence is selectively recognised by kallikrein, venom enzymes splitting substrates with this peptide residue with larger k_{cat}/K_M can be called kallikrein-like enzymes, or kininogenases (OHTANI *et al.*, 1988). The proteolysis of kallikrein substrate appeared to proceed under the action of young adders' venom by an order of magnitude slower than under the action of equal amount of adult adders' venom. The activity of venom was also decreased with respect to substrate I. It seems essential that the ratio of rates of proteolysis of these two substrates in young and adult adders is also different (which rules out the possibility that both substrates are hydrolysed by the same enzyme). Age dependencies of kininogenase activity of venom, determined by the hydrolysis of substrate II in mixtures 1 and 2 differing by the presence of the substrate

TABLE 2. AGE CHANGES OF *Vipera berus* VENOM*

Age† (years)	No. of individuals in group	Average amount of venom obtained from a bite, mg (relative amount as compared to the adult adder)	Rate of proteolysis ANSA substrates I and II ($P = 0.95$)‡				Kininogenase activity of a bite§
			I	II in mixture 1	II in mixture 2	II/I in mixture 1	
0	80	0.047 (0.015)	0.23 ±0.03	0.09 ±0.01	0.08 ±0.01	0.37 ±0.04	0.0013
1	8	0.72 (0.23)	0.85 ±0.11	0.67 ±0.07	0.51 ±0.06	0.78 ±0.10	0.16
2	6	1.11 (0.36)	0.97 ±0.12	0.70 ±0.09	—	0.72 ±0.09	0.25
3	10	1.77 (0.57)	0.91 ±0.12	0.70 ±0.09	0.80 ±0.10	0.77 ±0.10	0.40
4	6	2.67 (0.85)	0.99 ±0.13	0.75 ±0.10	0.83 ±0.11	0.75 ±0.10	0.64
5 and more	6	3.10 (1)	1	1	1	1	1

*Newly collected *Vipera berus berus* venom was obtained by a voluntary bite of a polyethylene covering draw on glass beaker. The venom obtained from a group of animals of one age was mixed. Animals of both sexes were present in each group. Snakes from the Tver population (the Tverskii region, Russia, is located 100–200 km north-west of Moscow) were captured 2 days prior to venom collection and kept at the Moscow Serpentarium. Venom was diluted with saline to an absorbance $A_{280\ nm} = 0.5$ ($d = 1$ mm) (concentration about 3.8 mg/ml).

†In natural populations newborn *V. berus* appear during a short time period at the end of summer. Therefore, a population consists of discrete age groups. Venom of newborns (age = 0) was collected several days after birth.

‡Values show the relative rate as compared to the adult adder.

§The kininogenase activity of a bite, measured as the multiple of the venom amount (column 3, in parentheses) and the rate of proteolysis of substrate II in mixture 1 (column 5).

listed second by efficiency, are similar (see Table 2, columns 5 and 6). This indicates that proteases relatively specific for both substrate I (the term 'thrombin-like proteases' used for Acyl–Gly–Pro–Arg-chromophore-cleft enzymes (TENG *et al.*, 1989)) and substrate II ('kininogenase') are present in viper venom. Interestingly, the ratio of proteolysis rates of substrates I and II gets stabilized by the first year of life, but then grows slightly once more in adult adders, as the activity levels of thrombin-like protease and kininogenase, typical for adults, are not achieved at the same time: in young adders (2–4 years old) the thrombin-like protease level is the same as in adult adders, while the level of kininogenase amounts to 0.7–0.8 that of adults.

As shown in Table 2, the average amount of venom (by protein) in a young adder's bite (0.047 mg against 3.10 mg dry weight) is more than 60 times smaller than that of an adult adder. The specific activities of proteases in a newborn adder's venom are reduced by a factor of 4.3 (the thrombin-like protease) and 12 (kininogenase). Thus, the amount of these (or substrate equivalent) proteases actually injected into blood by the bite of a newborn adder is several hundred times lower.

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