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PROTEINASES AND PHOSPHOLIPASES A-2 FROM THE VENOM OF *Vipera berus berus*

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With aid of chromatography combining gel filtration and ion-exchange methods, from the venom of the common adder Vipera berus berus *we have isolated proteinases and phospholipase A-2 and also a component with coagulating properties which, in contrast to the other proteinases of the venom, hydrolyzes casein feebly. According to the results of gel filtrations, the molecular mass of the coagulant isolated was about 100 kDa, pH 6.5-7.0. No phospholipase A-2 activity was found in the coagulant fraction.*

Snake venoms belonging to different systematic groups exhibit a considerable variability of composition and properties which is determined genetically and is connected with the physiological state of the producing animals and also with the methods of collecting and treating the venoms. Nevertheless, the simultaneous presence of a large number of different proteinases (EC 3.4.21-3.4.24) and phospholipases A-2 (EC 3.1.1.4) and the significance of just these enzymes in the overwhelming majority of biological effects has been reported as a common feature of the venoms of all viperid and crotalid snakes [1, 2]. Among the numerous and diverse effects of snake venoms, great interest is presented by their hemostatic action. In the present paper, the proteinases and phospholipases A-2 from the venom of the common adder *Vipera berus berus* are considered in connection with the influence of this venom on blood clotting.

The sample of the venom of the common adder *Vipera berus berus* that was used in our experiments had the characteristics given in Table 1.

For comparison, we give the results of analytical tests on the venoms of other viperid snakes: Renard's viper *V. ursini renardi* Ch. and the kufi, *V. lebetina turanica* Ch.

From the results of the tests used, the venoms investigated appeared fairly similar, the differences in proteolytic and phospholipase activities not reaching a large amplitude. Only the kufi venom stands out by a capacity for clotting plasma independently in the absence of calcium ions, while the other two venoms were active only in the recalcification test.

In the case of complex compositions such as snake venoms, the use of tests characterizing a material in a generalized fashion often does not give a complete idea of their actual possibilities, which are hidden as the result of a mutual interference of the biologically active components. Separation into components is then the most suitable method for discovering and exploiting new properties.

On the gel Sephadex G-75, the whole venom of the common adder was separated into a considerable number of fractions (up to 10), but because of the unsatisfactory separation of some of them we first collected five fractions (I–V) corresponding mainly to the position of a proteinase (I and II), of a phospholipase A-2 (II and III), and of low-molecular-mass components (IV and V) where the proteinase activity was slight and phospholipase A-2 activity was absent (Fig. 1A). All the fractions obtained were heterogeneous and, in accordance with the theory of gel filtration, the concentration of low-molecular-mass components in them increased in order of their emergence from the column. The ratio of the column parameters and the elution volumes enabled the molecular masses of the components to be estimated as follows: I — above 60 kDa; II — 40-30 kDa; III — 30-10 kDa; IV and V — below 10 kDa. The protein content of the adder venom was 86%, the bulk of the proteins issuing in fractions I, II, and III (25.92, 15.02, and 41.95%, respectively), which together make 82.89%. The remaining 17.11%

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TABLE 1. Enzymatic Activities and Biological Effects of the Venoms of *V. berus (1), V. ursini renardii* Ch (2), and *V. lebetina turanica* Ch. (3)

Index	1	2	3
1. Toxicity (LD-50), mg/kg body weight of i/p injection	0.84	2.60	1.86
2. Clotting time, sec	Abs.	Abs.	144.0
3. Recalcification time, sec*	32.0	22.0	17.0
4. Proteolytic activity, units/mg of venom	2.34	3.65	6.10
5. Phospholipase A-2 activity, min	20.0	30.0	20.0

*Recalcification time in control (U0-160 sec.).

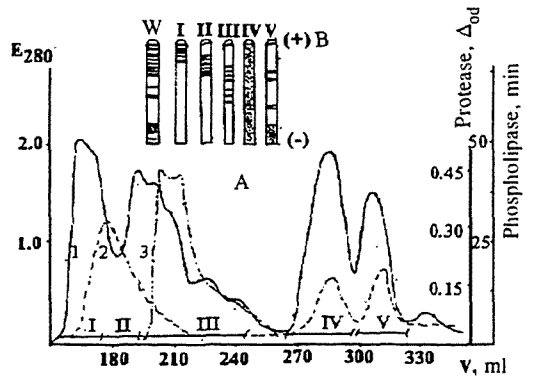

Fig. 1. Graph of the separation of the venom of the common adder (300 mg) on Sephadex G-75. The column (25 × 930 mm) was equilibrated with 0.05 M ammonium acetate buffer, pH 6.8. Elution with the equilibrating buffer at the rate of 30 ml/h. A) 1 Absorption at 280 nm (protein content); 2) proteolytic activity; 3) phospholipase A-2; I-V) preparatively sorbed combined fractions; B) electrophoregram of the whole venom (W) and of the preparative fractions I-V.

was accounted for by fractions IV and V and also included losses of material due to the nonspecific adsorption of the proteins on the dextran gel.

The distribution of the main enzymatic activities — the proteolytic and phospholipase activities — over the fractions was the usual one for the gel filtration of the venoms of viperid snakes: possessing smaller dimensions, the phospholipase A-2 was separated from the proteinases, which have larger molecular dimensions [3, 4]. However, the use of Sephadex G-75 permitted a more effective exclusion of the proteinases, because of which their separation from the phospholipase A-2 was improved. The proteins from the zone of high proteolytic activity (fractions I and II) shortened the recalcification time of citrate plasma, which corresponded to the coagulant properties of the whole venom. Conversely, proteins lengthening the recalcification time were present in the phospholipase A-2 zone, the role of such anticoagulant being claimed by the phospholipase A-2 itself. Thus, after fractionation it was possible to detect previously hidden properties of the venom and components with an anticoagulant action corresponding to them.

The anticoagulant action of phospholipases A-2 from the venoms of viperid snakes has been decribed previously [5, 6]. However, not all phospholipases A-2, even within a single venom, possess an anticoagulant action. Thus, a second gel filtration of the proteins of fraction III, where the phospholipase and anticoagulant effects of the venom were revealed, permitted a better separation of the already-labeled protein peaks (Fig. 1,A): phospholipase A-2 was detected over the whole separation but with a maximum in fraction III-2, while anticoagulant properties were found only in fraction III-1 (Fig. 2A). Such noncoincidence in the distribution of the activity maxima permitted the assumption of the presence in common adder venom of specific enzymes with anticoagulant properties in addition to phospholipases A-2. However, the results of electrophoresis (Fig. 2,B), witnessing the heterogeneity of the fractions obtained and revealing in fractions III-1 and III-2 com-

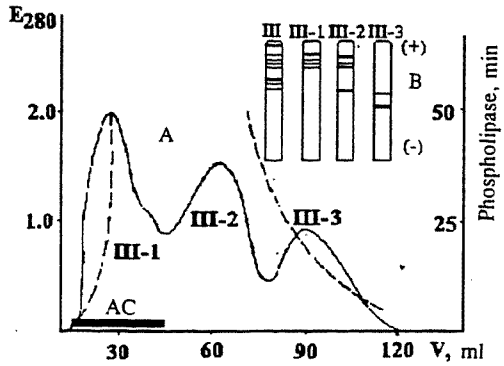

Fig. 2. Graph of the separation of fraction III on Sephadex G-75. For the conditions of gel fltration, see Fig. 1A. A) 1) Absorption at 280 nm (protein content); 2) coagulation time of egg yolk (phospholipase A-2); AC) anticoagulant zone; III-I–III-3) fractions combined according to the protein peaks; B) electrophoregrams of the initial fraction III and the subfractions III-1–III-3 obtained from it.

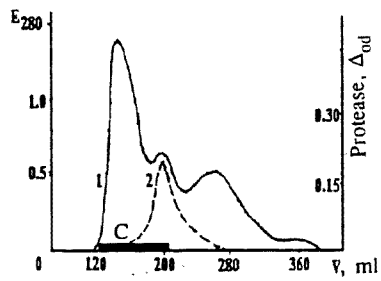

Fig. 3. Graph of the separation of fraction I-II on Sephadex G-75. For the conditions of gel filtration, see Fig. 1A. 1) Absorption at 280 nm (protein content); 2) proteolytic activity; C) zone of the coagulant.

ponents with overlapping molecular masses, do not exclude the possibility of the existence in the adder venom and, consequently, in fractions III and III-1 of an independent anticoagulant not connected with phospholipase A-2. Since a large number of anticoagulants belonging to the proteinase group are found in snake venoms, the presence of proteins with overlapping dimensions in the zones of phopholipase A-2 (III) and of the proteolytically active fraction II (Fig. 1,A) may be evidence in favor of this hypothesis.

We also analyzed the link between the coagulant properties of the venom and its proteolytic action with the aid of repeated gel filtration. On the chromatographic profile of combined fraction I-II focusing the whole mass of the caseinolytic enzyme and the coagulating properties of the venom, a shift of the enzymatic activity concentrated in fraction I-2 relative to the coagulating activity found in fraction I-1 is clearly seen (Fig. 3). Thus, if the coagulating component of the venom is also the proteinase component it differs in specificity from a caseinase. Chromatography on the ion-exchange resin CM-32 (results not given) confirmed this conclusion: in the chromatographic process, a component with pronounced coagulant properties but inferior to the other fractions in proteolytic activity on casein was isolated from several fractions effectively hydrolyzing casein. According to the results of gel filtration, the molecular mass of the coagulant was about 100 kDa, and according to the conditions of its desorption from the cation-exchange resin and isoelectric focusing its pI was 6.5-7.0. On fractionating common

adder venom, M. Yu. Samel [7] also found a distribution of casein-hydrolyzing activity over several fractions. From its molecular parameters, the coagulant that we obtained corresponded most closely to the proteinase of fraction II, characterized by a molecular mass of 100 kDa and pI 6.1 [7].

EXPERIMENTAL

The venom of the common adder *Vipera berus berus* dried in a desiccator over calcium chloride was purchased from the Central Asian Zonal Zoological Combine; a batch of venom from the 1985-1990 collection was stored in vacuum packing in the cold. We used Sephadexes G-75 (medium) and G-25 (fine), substances with standard molecular dimensions produced by Pharmacia (Sweden), carboxymethylcellulose CM-32 from Whatman (United Kingdom), a set of reagents for electrophoresis from Reanal (Hungary), and other reagents of domestic production of grades kh.ch ("chemically pure") or ch.d.a ("pure for analysis"). Casein was prepared in accordance with the recommendations of [8], and an emulsion of egg yolk was diluted with phosphate-buffered (pH 7.0) physiological solution in a ratio of 4:5 (v/v) [9].

White mice were purchased from Zootek (Tashkent).

Proteolytic activity was determined from the hydrolysis of casein [10], taking as the unit of activity the amount of enzyme increasing the optical density of the incubation medium by 0.1. Phospholipase A-2 was estimated from the time of coagulation of an emulsion of egg yolk, the unit of activity corresponding to the clotting time in minutes [9]. The tests for blood clotting were performed in accordance with known recommendations [11]. Toxicity (LD-50) was evaluated after the intraperitoneal injection of the material obtained into white mice weighing 18-20 g. The results obtained were treated statistically by the Litchfield–Wilcoxon method [12].

Gel filtration on the Sephadexes, chromatography on ion-exchange resins, and isoelectric focusing on PAAG plates with Ampholines were conducted in accordance with the recommendations of the manufacturers (Pharmacia, Sweden, and Whatman, United Kingdom), while disk electrophoresis was carried out by Laemmli's method [13].

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REFERENCES

1. D. N. Sakhibov, V. M. Sorokin, and L. Ya. Yukelson, The Chemistry and Biochemistry of Snake Venoms [in Russian], Fan, Tashkent (1972).
2. T. Iwanaga and T. Suzuki, Enzymes in Snake Venoms. Vol. 52 of Handbook of Experimental Pharmacology, Springer, Berlin (1979), p. 61.
3. E. Siigur, J. Siigur, M. Nommeots(Samel), and T. Ilomets, Toxicon, **17**, No. 6, 623 (1979).
4. M. Samel, E. Siigur, and J. Siigur, Toxicon, **25**, No. 4, 379 (1987).
5. M. C. Boffa, C. Rothen, H. M. Verheij, R. Verger, and G. H. De Haas, in: Natural Toxins, D. Eaker and T. Wadström (eds.) Pergamon, Oxford (1980), p. 131.
6. G. E. Lyubimtseva and L. Ya. Yukelson, Khim. Prir. Soedin., 399 (1983).
7. M. Yu. Samel, Author's abstract of dissertation ... Candidate of Biological Sciences [in Russian], Tallin (1987).
8. G. Sarath, R. S. De La Motte, and W. Wagner, in: Proteolytic Enzymes, A Practical Approach, R.-J. Beynon and J. S. Boed (eds.), IRL Press, Oxford (1989), p. 25.
9. E. Habermann and W. Neumann, Hoppe-Seylers Z. Physiol. Chem., **197**, No. 3-6, 179 (1954).
10. L. P. Alekseenko, in: Modern Methods in Biochemistry [in Russian], V. N. Orekhovich (ed.), Meditsina, Moscow (1968), p. 115.
11. V. P. Baluda, Z. S. Barkagan, E. D. Gol'dberg, B. I. Kuznik, and K. M. Lakin, in: Laboratory Methods of Investigating the Hemostasis System [in Russian], E. D. Gol'dberg (ed.), Tomsk (1980).
12. M. L. Belen'kii, Elements of the Quantitative Estimation of a Pharmacological Effect, GIML, Moscow (1963).
13. U. K. Laemmli, Nature (London), **227**, 680 (1970).