AN INVESTIGATION OF THE COAGULOLOGICAL POTENTIAL OF THE VENOMS OF SOME CENTRAL ASIAN SNAKES

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The compositions of the proteolytic complexes of the venoms of the crotalid Agkistrodon halys halys *and the viperid* Echis multisquamatus *have been investigated with the aid of a large number of specific natural and model substrates. A comparative analysis of the results obtained has revealed a predominance in the crotalid venom of fibrinogen-hydrolyzing enzymes, while the viperid venom is characterized as a procoagulant of the prothrombin-activating type. Among the fibrinogenases of the crotalid venom, a thrombin-like enzyme and a plasmin-like proteinase have been revealed and obtained in a purified state. A prothrombin-activating enzyme from the viperid venom has been isolated in the purified state and characterized with the aid of specific substrates.*

Medical coagulology is a promising field for the practical use of snake venoms and preparations based on them [1]. Questions of the use of venoms and their components as diagnostic or corrective agents have been considered previously in the domestic and foreign literature [2, 3], but this general discussion cannot replace a concrete analysis of individual snake venoms in the application to this problem. The consideration of the latter is further complicated by the fact that the venoms include components with mutually exclusive reactions, and the results of a study of whole venoms are represented by integral characterizations masking their true possibilities. Such interference can be overcome by the use of specific substrates and by the creation of reactions conditions oriented to revealing strictly determined components or by the fractionation of the venom. It is just these approaches that we have used in the present work for the analysis of the coagulological potential of the venoms of *Agkistrodon halys halys* and of the *Echis multisquamatus*, representatives of the families of rattlesnakes (Crotalidae) and vipers (Viperidae) living in the Central Asian region.

The importance of proteinases in the cascade of blood clotting and fibrinolysis reactions is known [4]. Table 1 gives information characterizing the action of both the venoms investigated on various natural protein–peptide substrates and also on model compounds the hydrolysis of which permits the revelation of enzymes specific with respect to the site of their action. The greatest possible number of different specific substrates was investigated with a limitation to a qualitative evaluation ("+") or ("–") in those cases where we did not have a sufficient amount of the substance available.

Although because of the nonstandard nature of snake venoms quantitative estimates of their parameters are extremely relative, the facts given (Table 1) reveal fairly substantial differences in the compositions of the proteolytic complexes formed by them. These differences are expressed clearly only in a comparative analysis of the action of the venom on specific substrates (S-2337, –2366, –2251, Chromozyn PL), and different directions of coagulological action correspond to them (Table 2): the crotalid venom is more effective with the substrate of the final reaction of the cascade of fibrinogen, while the

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TABLE 1. Proteolytic Activities of the Venoms of the Crotalid *Agkistrodon halys halys* and the Viperid *Echis multisquamatus*

Substrate	Activity, units/mg of venom protein	
	crotalid	viperid
1. In the hydrolysis of natural substrates:		
hemoglobin (pH 2.65)	6,50	0,67
casein (pH 8.0)	11,50	4,89
dimethylcasein (pH 8.6)	67,50	31,11
kininogenin (pH 8.0)	0,008	0,0066
bradykinin	—	—
2. In the hydrolysis of model substrates:		
BAEE	1,88	4,11
ATEE	1,50	3,60
BAPNA	—	—
Hyppuryl-L-arginine	0,12	0,27
chromogenic compounds (S):		
2238	0,81	6,67
2337	0,06	0,41
2366	0,16	0,056
2302 or Chromozym PK	7,96	9,15
2251	0,38	0,071
Chromozym PL	+++	+

TABLE 2. Characteristics of the Action of the Venoms of the Crotalid *Ag. halys halys* and the Viperid *E. multisquamatus* on Blood Coagulation and Fibrinolysis

Characterizing tests	Venoms	
	crotalid	viperid
1. Coagulation time of citrated plasma, sec*		
a) without calcium	25,0 + 2,0	16,0 + 2,0
b) with calcium (recalcification).	20,0 + 1,0	10,0+1,0
2. Fibrinolysis (lysis of fibrin plates), mm^2		
a) heated at 75°C	110	+ −
b) unheated	256	+ −
3. Coagulation time for pure fibrinogen, sec		
a) in the presence of the whole venoms	120 **	—
b) on the addition of thrombin after the venoms	—	Fine suspension
4. Hydrolysis of coagulation substrates, nmole/min/mg of venom		
a) prothrombin	0,006	187,4
b) factor X	0,002	0,36
c) factor Y	0,025	0,24

*In the coagulogical tests the venoms were used in a concentration of 100 μg/ml.

**Weak coagulum passing into a fine suspension forming turbidity.

viperid venom is more active in the early stages of coagulation. This conclusion, based on a comparison of the efficacy of the hydrolysis of specific model substrates also follows from results characterizing the action of the venoms on purified natural venoms on purified natural substrates (fibrinogen, prothrombin, factors X and Y) but not from general coagulogical tests.

Thus, the use of selective test systems permits an unambiguous determination of procoagulant properties for the viperid venom, while the crotalid venom is characterized by a high efficacy in relation to fibrogenin. In this connection, the results relating to the hydrolytic action of the venoms being compared on the chromogenic substrate S-2238, which is specific for thrombin, appear of interest. While the high activity of the viperid venom in relation to the substrate can be explained by its content of a corresponding proteinase which, however, is ineffective on the natural substrate, the low activity of the crotalid venom requires further study. In order to obtain more detailed information, we had recourse to the fractionation of the venoms.

TABLE 3. Hydrolytic Action of the Crotalid Venom and Its Fractions on the Chromogenic Substrates

Venom and fraction	Hydrolytic activity, nM of p-NA/min/mg in the hydrolysis of substrates (S)				
	2238	2337	2366	2302	2251
Whole venom	0,81	0,06	0,16	7,9	0,38
CS	0,98	0,09	0,26	20,7	0,58
CM-9 TLE	2,61	0,11	0,64	13,2	0,22
ACS	0,78	0,10	0,25	2,7	0,54
C2	0,92	0,21	0,30	3,1	0,65
C3	1,06	0,14	0,39	2,6	0,86

pNA — para-nitroaniline.

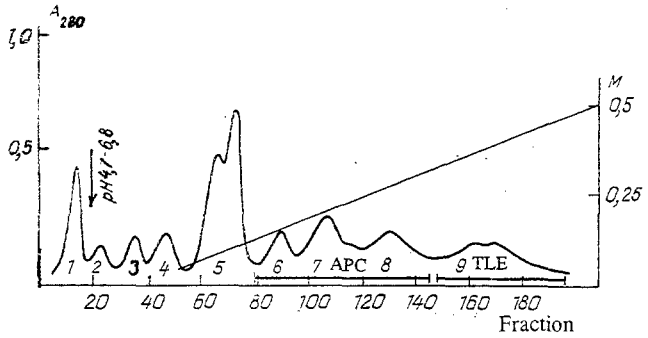

Fig. 1. Graph of the chromatography of the proteins of the coagulant section (CS) of the crotalid venom on CM-cellulose. Columns (1.8 × 35 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 4.7). Absorption in the equilibrating buffer. Desorption with a linear gradient of pH (4.7-6.8) and concentration (0.05-0.3 M) of the equilibrating buffer. CM-1−CM-9) Fractions combined according to the protein "peaks." The zones of the thrombin-like enzyme (TLE) and the activator of protein C (APC) are marked.

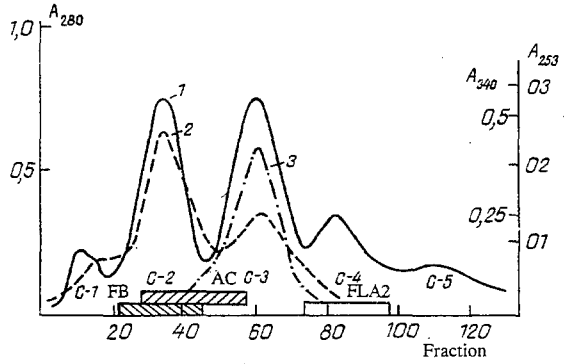

Fig. 2. Graph of the gel filtration of the proteins of the anticoagulant section (ACS) of the crotalid venom on Sephadex G-75 Column (2.5 × 160 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 7.6). Elution with the equilibrating buffer. C1−C4) Fractions combined according to the protein "peaks." 1) Absorption at 280 nm (concentration of protein in the sample); 2) Proteolytic activity from the hydrolysis of casein; 3) BAEE esterase activity. The anticoagulant (AC), fibrinolysin (FB), and phospholipase A-2 (PLA-2) zones are indicated.

TABLE 4. Enzymatic Activities and Coagulological Characteristics of the Fractions of the Anticoagulant Components of the Crotalid Venom

Substances investigated	Activities of the enzyme, units/mg of protein in the venom			Coagulological characteristics	
	proteinase (casein)	BAEE esterase	Phospho-lipase A-2	Anticoagulant properties (recalcification, sec)	Fibrinolysis, mm^2
ACS	12,0	3,0	12300	>300 + 3	255
Fraction					
C1	—	—	—	170* + 2	42
C2	3,0	—	—	>300 + 3	156
C3	1,0	6,0	350	260 + 3	80
C4	—	—	14800	185 + 3	—

*Time for the recalcification of citrate plasma in the control 175 ± 2 sec.

FRACTIONATION OF THE *Agkistrodon halys halys* VENOM

By chromatography on DEAE−Sephadex (or DEAE cellulose), the whole crotalid venom was separated into a number of fractions which, on the basis of general coagulogical tests (recalcification, coagulation of purified fibrogenin, fibrinolysis) were grouped into two sections: a coagulant section (CS) containing mainly the basic components of the venom, and an anticoagulant section (ACS) in which the more acidic proteins and peptides were concentrated [5-7]. The CS differed fundamentally from the remainder of the venom not only by the stimulation of the coagulation of citrate plasma but also by its capacity for forming stable fibrogen clots; the addition of the ACS blocked this action of the CS (just like that of thrombin!) and practically restored the effect of the whole venom, when we observed the production from purified fibrogenin of an unstable clot rapidly breaking down to a protein suspension forming a turbid solution. It was possible to separate the thrombin-like activity from part of the ballast and to focus it within a single fraction by chromatographing the CS on CM-cellulose (Fig. 1). Although the thrombin-like fraction (TLF) retained the capacity for hydrolyzing a chromogenic substrate of activizing protein C (S-2366), experiments with the natural substrate showed a separation of the corresponding activity from the TLF. Table 3 shows the rise in the efficiency of the hydrolytic action of the latter on the thrombin-specific substrate S-2238 against a background of falling activity in relation to the plasmin substrate S-2251. The hydrolysis of the model substrates S-2238 and S-2251 imitates the action on the natural substrate of fibrinogen. The efficacy of the hydrolysis of these model substrates by the CS and the ACS were comparable but they differed considerably with respect to the nature of their action on fibrinogen, since the ACS and fractions C2 and C3 obtained from it (Fig. 2) not only did not coagulate purified fibrinogen but actually prevented the coagulating action of the CS, the TLF, and thrombin.

As one of the alternatives explaining the results obtained it is possible to assume the presence in the ACS of a proteinase of the plasmin type (fibrinogenase) in contrast to the TLE, which hydrolyzes fibrinogen in such a way as to interfere with its capacity for polymerizing.

In the experiments with pure plasminogen, the ACS and fractions C1−C4 did not cause the formation of plasmin. Other properties of the components of the ACS are shown in Table 4. By gel filtration on Sephadex it was possible to isolate the phospholipase A-2 (C-4) and, by directed testing, to exclude it from the number of possible anticoagulants. The C1 fraction, possessing the insignificant fibrinolytic effect, did not change the control indices, either. The role of anticoagulant in the ACS is claimed by the components C2 and C3 of the section, although the latter proved to be less powerful in both tests: recalcification and fibrinolysis. The mutual impurities (the fractions were electrophoretically heterogeneous) made an accurate identification of the anticoagulant difficult; however, it may be characterized in preliminary fashion as a proteinase hydrolyzing casein but inactive in relation to arginine esters (BAEE); this proteinase is a powerful fibrinolysin, probably of the plasmin type, since it did not activate plasminogen.

On the whole, the results obtained have revealed in the crotalid venom, factors that, of the coagulation and fibrinolysis substrates, attack mainly fibrinogen: from the site of action in fibrinogen it is possible to differentiate the thrombin-like enzyme — the main active principle of the CS — and a casein-hydrolyzing proteinase which causes the destruction of fibrinogen as a result of which it loses its capacity for polymerizing; it is probably the main active principle of the ACS, and its accurate

TABLE 5. Enzymatic Activities and Coagulological Characteristics of Fractions of the Anticoagulant Components of *Ag. halys halys* Venom

Venom and fraction	Hydrolytic activity, nmole of p-NA/min/mg in the hydrolysis of substrates (S)				
	2238	2337	2366	2302	2251
Whole venom	6,67	0,41	0,056	9,15	0,071
Fraction VI	33,58	1,10	0,059	25,79	0,145
Increase in specific activity (activity of the fractions/mg)	5,03	2,68	1,05	2,81	2,04

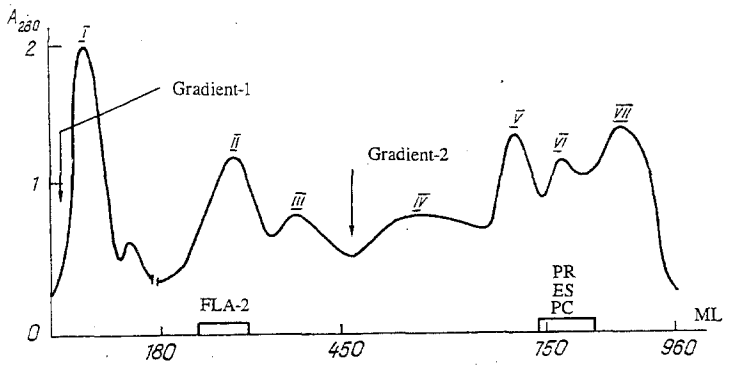

Fig. 3. Ion-exchange chromatography of the venom of *E. multisquamatus* on CM-Sephadex C-50. Column (2.5 × 10 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 5.5). Elution: gradient 1: acetate, pH 5.5-0.05 M ammonium acetate, pH 8.0; gradient 2: 0.05 M ammonium acetate, pH 8.0-0.5 M ammonium acetate, pH 8.0. I-VII: fractions collected and combined in accordance with the protein "peaks"; PLA-2) phospholipase A-2; PR) proteinase; ES) esterase; PC) procoagulant.

identification will be possible only after its isolation in the pure form and substrate analysis using specific models and natural substrates.

FRACTIONATION OF THE *Echis multisquamatus* VENOM

The high activity of the viperid venom in relation to the specific thrombin substrate S-2238 does not correlate with the low efficacy of the thrombin-like action of this venom; judging from the hydrolysis of S-2251, its plasmin-like activity was not high, either (Table 1). At the same time, in full agreement with literature information [8], this venom actively attacked substrate S-2337, which is specific for factor Xa, and prothrombin (Tables 1 and 2), forming typical intermediates [9]. In order to characterize the active principle in more detail, the whole venom was chromatographed on the cation-exchange resin CM-Sephadex C-50 (Fig. 3).

Of the seven fractions (I-VII) obtained, fraction VI possessed proteolytic (PR) activity with respect to the hydrolysis of protein, BAEE esterase (ES) activity, and procoagulant (PC) properties, since it shortened the time of recalcification with the absence of a visible effect on fibrinogen. It appeared of interest to characterize this fraction in comparison with the initial venom in terms of its action on specific chromogenic substrates (Table 5). A dissimilar rise in specific activity in the sequence of substrates investigated showed that fraction (VI) contained several proteinases with different substrate specificities. One of them was active on a model substrate (2238) but did not attack the corresponding natural substrate — fibrogenin; after treatment with another proteinase (venom, fraction VI), fibrinogen lost its capacity for giving a coagulum in the presence of thrombin (Table 2), but the plasmin activities of the venom and of fraction VI were extremely low in comparison with the venom and fractions from the crotalid. Of course, it is not excluded that some enzymes of the venoms are weakly specific in relation to the amides of arginine or of lysine and therefore attack a wide range of substrates. However, the presence in the venom and

in fraction VI of a fibrinogenolysin (proteinase) with low activity on the model substrate of plasminogen is more likely. Such fibrinogenolysins have been found previously in the preparation Ecarin — an Xa-like proteinase analogous to the enzyme present in the venom of the viperid *E. multisquamatus* and fraction VI [10, 11]. As in the case of the crotalid venom, only the isolation of the required agent in the pure form and subsequent substrate analysis will enable it to be given an accurate definition.

EXPERIMENTAL

The venom of the crotalid *Ag. halys halys* and the viperid *E. multisquamatus*, dried in a desiccator over calcium chloride, 1989-1991 collections, were purchased from the Institute of Zoology and Parasitology. We also used bradykinin, casein, hemoglobin, the ether esters of N-benzoyl-L-arginine (BAEE) and of acetyl-L-tyrosine (ATEE), benzoylarginine p-nitroanilid (BAPNA), and hippuryl-L-arginine produced by Reanal (Hungary), fibrogenin and thrombin from the Kaunass Bacterial Preparations Factory, Lithuania), plasminogen and the chromogenic substrates S-2238, S−2237, S−2366, S−2251, and S−2302 from AB Kabi Diagnostica (Sweden), Chromozym PK and L from Boehringer Mannheim Gmbh (Germany), p-nitrophenyl p-guanidinobenzoate (p-NPGB) from Nutritional Biochemicals Inc. (USA), and *V. russelli* venom, phosphotidylserine, and phosphatidycholine from Sigma (USA).

Casein was methylated by the method of Lin et al., [12], and human and bovine factors Xa [15, 16] were obtained independently. The molar concentrations of prothrombin and of factor X were determined after their complete activation by the venoms of *E. multisquamatus* and *V. russelli*, respectively, and the titration of the active centers with p-NPGB [17, 18], and factor Y after its complete activation by thrombin [19]. An emulsion of egg yolk was prepared independently in physiological solution buffered with phosphate to pH 7.0 [20]; the ratio of phospholipids and physiological solution was 4.5 (v/v). In the chromographic and electrophoretic investigations we used Sephadexes and DEAE- and CM-Sephadexes from Pharmacia (Sweden), DEAE- and CM-celluloses from Serva (Germany), and kits of reagents for disk electrophoresis from Reanal (Hungary).

Proteolytic and esterase activities were determined by known methods [21, 22]. Amidolytic activities in relation to the chromogenic substrates were measured at 37°C in a medium containing 50 mM Tris HCl (pH 7.9), 175 mM NaCl, 2 mM calcium chloride, and 200 μM substrate; the amount of p-nitroaniline was calculated by using a molar extinction coefficient for it of 104,100 $mole^{-1}/liter/cm^{-1}$. The activity of phospholipase A-2 was investigated on the basis of the inhibition of the coagulation of egg yolk [20] and titrimetrically [23]. Coagulological tests were performed in accordance with the recommendations of V. P. Baluda, et al. [24], and the activation of the coagulation factors was investigated in the way described previously [8]. Protein concentrations were determined spectrophotometrically and by Lowry's method [25]. Chromatography and gel filtration were carried out in accordance with the recommendations of the manufacturers of the sorbents and molecular sieves, and electrophoresis was conducted by Laemmli's method [26].

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