VENOM OF THE MAMUSHI *Agkistrodon halys halys.*

IV. THROMBIN-LIKE PROTEASES

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UDC: 547.993

An enzyme similar thrombin forming a fibrin clot on addition to citrate plasma or solutions of purified fibrinogen and called a thrombin-like protease (TP) was detected after the separation of the whole venom of the mamushi *Agkistrodon halys halys* into fractions with the aid of gel filtration [1]. TP can also be revealed by the chromatography of the whole mamushi venom on DEAE-Sephadex A-50 or other anion-exchange resins, the distributions of its activity being followed throughout all the so-called "coagulant" fractions characterized by a predominant content of the main components of the venom. For the further purification of the TP, the fractions obtained from the anion-exchange resin were subjected to chromatography on the cation-exchanging CM-cellulose equilibrated with 0.05 M ammonium acetate uffer (pH 4.7). Under these conditions, part of the components was not bound to the CM-cellulose but issued from the column in three protein "peaks." Subsequently, when the concentration and pH of the eluent were changed from 0.05 to 0.5 M and from 4.7 to 6.8, desorption of the remaining components as seven protein "peaks," which we collected preparatively, was observed. The results of the biological testing of all ten fractions, CM_1-CM_{10} are given below (the statistically significant mean values of not less than six measurements are given):

Fraction	BAEE-esterase, μmole/mg/min	Caseinase, units/mg	TP, units/mg	Recalcification time of citrate plasma, sec
CM_1	0.606	—	—	150
CM_2	0.819	—	—	80
CM_3	0.322	—	—	190
CM_4	7.145	—	—	105
CM_5	0.929	0.6	—	245
CM_6	1.774	1.0	—	155
CM_7	2.024	1.3	—	140
CM_8	1.921	1.1	—	110
CM_{9-10}	7.739	—	285	1–2
Control	—	—	—	115

In the case of chromatography on CM-cellulose, the thrombin-like activity was concentrated within the two fractions CM_9 and CM_{10}, which we combined into one, CM_{9-10}. To purify the TP of this electrophoretically heterogeneous fraction, as variants we peformed its gel filtration on Sephadexes G-75 and G-100 and its chromatography on the anion-exchange resins TEAE-cellulose and QAE-Sephadex A-50.

The best separation of the CM_{9-10} fraction was achieved on QAE-Sephadex A-50 equilibrated with 0.01 M ammonium acetate (pH 8.2). Part of the components of the fraction was not adsorbed on the anion-exchange resin and issued with the eluting solution as five protein "peaks" (Q_1- Q_5); 0.25 M ammonium acetate solution desorbed another two fractions, Q_6 and Q_7. All these fractions except Q_5 had thrombinlike activities of different efficiences and shortened the recalcification time; the most powerful activity with respect to the two parameters studied was possessed by the fractions Q_1 and Q_2. Disk electrophoresis and isoelectric focusing indicated the individuality of the TPs of these fractions. Additional proofs of the homogeneity of fraction Q_1 were obtained on SDS-electrophoresis, analysis of the amino acid composition and of the N-terminal amino residue, and the absorption spectrum. The results of physicochemical analysis showed that one of the TPs detected in the mamushi venom (Q_1) was characterized by a molecular weight of 30-31 kD and by pronounced

Institute of Biochemistry, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 850-851, November-December, 1985. Original article submitted July 11, 1985.

0009-3130/85/2106-0817-$09.50

basic properties (pI 9.5) and contained valine at the N-end.

The possibility of the existence of multiple forms of TP in mamushi venom is shown by the results of the chromatography of the venom and its fraction on anion-exchange resins, revealing a large number of individual fractions with thrombin-like activity, and by the isolation of two individual TPs differing in the values of their isoelectric points. At the same time, in connection with our experiments performed in TP fractions homogeneous with respect to protein, the presence of sugars (8-10%) may show that the heterogeneity of the population of TPs in mamushi venom is explained by the "certain degree of natural polydispersity" that is characteristic of glycoproteins in general [2].

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VENOM OF THE MAMUSHI Agkistrodon halys halys.

V. COMPONENTS WITH AN ANTICOAGULANT ACTION

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UDC: 547.993

By chromatography on DEAE-Sephadex A-50, the venom of the mamushi Agkistrodon halys halys can be separated into two groups of fractions, of which one contains mainly the basic and the other the acidic protein components of the whole venom. The first group of fractions of the basic components is characterized by a capacity for stimulating the clotting of blood and is defined as the "coagulant fraction" of the venom. Conversely, in the presence of the components of the second group of fractions the clotting time is extended.

In these fractions of the acid components of the venom forming its "anticoagulant fraction," we have detected a phospholipase A_2 and substances lysing heated and unheated fibrin plaques. We have also shown that in the presence of the components of the anticoagulant fraction of the venom thrombin and the thrombin-like proteases isolated from the coagulant fraction from the mamushi venom lose their capacity for forming a fibrin clot in solutions of purified fibrinogen or in plasma.

In the subsequent experiments we attempted to identify and make a preliminary characterization of the active principle (or principles) of the anticoagulant fraction of the mamushi venom. Figure 1 gives the results of the gel filtration and ion-exchange chromatography of the fraction of acid components of the mamushi venom. When various molecular sieves were used (Acrilex, Bio-Gel, Sephadex) it was possible to separate a phospholipase A_2 from a protein anticoagulant with an antithrombin action. The phospholipase A_2 itself, which, with some modification of the purification procedure, can be obtained in the individual state, had a molecular weight of about 20 kD and a pI of 4.7, i.e., it was an acid phospholipase A_2. Like other acid phospholipases A_2 of snake venoms [1], it possessed no anticoagulant action. The same separation of the protein with antithrombin activity and the phospholipase A_2 was achieved with the aid of ion-exchange chromatography on CM-cellulose.

Thus, the phospholipase A_2 that we isolated in the pure form must be eliminated from among the probable anticoagulants of the mamushi venom while, as the active principle ensuring the anticoagulant properties of the venom, attention is attracted by a protein with the antithrombin properties and by proteins present in all the fractions with a direct and

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Institute of Biochemistry, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 851-852, November-December, 1985. Original article submitted July 23, 1985.

 0009-3130/85/2106-0818$09.50