PHOSPHOLIPASE ACTIVITY OF THE VENOM OF *Echis multisquamatus*

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Of the two molecular forms of phospholipase A_2 detected in the venom of the viper *Echis multisquamatus*, one, with a molecular weight of 15 kD, has been obtained in the pure form.

The overwhelming majority of snake venoms contain phospholipases A_2 which differ substantially from one another in their physicochemical properties and, correspondingly, in their biological action [1, 2]. Frequently, the phospholipases A_2 are present in snake venoms in multimolecular forms [3, 4] having not only differences in structure but also differences in substrate specificity. The identification of and subsequent study of phospholipases A_2 present in a definite snake venom is therefore an extremely interesting and at the same time absolutely independent problem.

Like all venoms of snakes of the family *Viperidae*, the venom of the *E. multisquamatus* is "high-molecular," i.e., the bulk of it consists of substances with relatively large molecular dimensions. It was possible to convince ourselves of this on the basis of the results of gel filtration of this venom. On passage through a column of Sephadex G-75, the viper venom was separated into seven fractions (Fig. 1a). Because of the insufficiently good separation of the individual protein peaks from one another during separation, we collected only three fractions, denoted in the order of their issuing from the column as I, II, and III. The bulk of the protein was concentrated in the first and second fractions (80.6 and 59.9 mg, respectively), while the low-molecular-weight fraction III contained only 13.3 mg of protein. The losses of material, amounting to 70.7 mg of protein, are partially explained by the elimination of mucus, epithelia cells and other ballast.

Of the fractions shown in Fig. 1a the first contained high-molecular-weight components of the venom and possessed no phospholipase A_2 activity. The third fraction consisted of a mixture of comparatively small peptides, free amino acids, and other components, with molecular weights of less than 5000 and was characterized by a low phospholipase activity.

The greatest interest was presented by the second fraction, which contained practically all the phospholipase A_2 activity of the whole venom. According to the theory of gel filtration [5], this fraction should have contained components with molecular weights in the range of 10,000-25,000 daltons, which corresponds to literature information on the molecular dimensions of phospholipases A_2 from venoms of snakes of the *Viperidae* family [1].

The results of comparative electrophoretic investigations of the venom of *E. multisquamatus* and its fractions obtained after separation of Sephadex G-75 showed their heterogeneity (see Fig. 1a). Subsequently, 20 mg of fraction II was dissolved in 10 ml of 0.05 M ammonium acetate buffer with pH 4.7 and was deposited on a column of SP-Sephadex equilibrated with the same buffer. Part of the fraction (component II-1) was not adsorbed under these conditions and issued in the starting buffer. The further desorption of the protein components was carried out with a linear pH gradient (from 4.7 to 9.1) of 0.05 M ammonium acetate buffer, giving three components: II-2, II-3, and II-4. When the molarity of the ammonium acetate buffer (pH 9.1) fed to the column was increased to 0.5 M it was possible to obtain one more fraction, II-5. Thus, with the aid of ion-exchange chromatography on SP-Sephadex G-25, fraction II was separated into five components: II-1—II-5 (Fig. 1b).

Determinations of phospholipase activity showed that the enzyme was localized only in fraction II-3, desorbed from the ion-exchanger at pH 5.4-5.7 of the eluting solution. The yield of the phospholipase A_2 fraction amounted to 3.84%.

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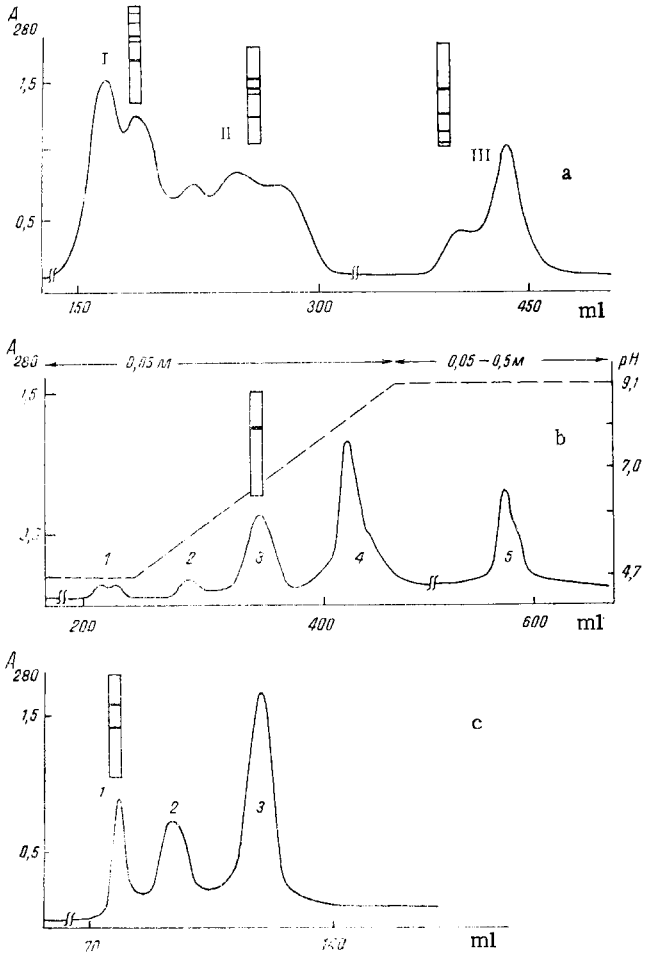

Fig. 1. Isolation of phospholipases A_2 from the venom of the *Echis multisquamatus* (conditions of the experiments in the text): a) fractionation of the whole venom on Sephadex G-75; b) separation of fraction II on SP-Sephadex; c) separation of fraction III on Sephadex G-25.

Disk electrophoresis in acid and alkaline media revealed the individuality of fraction II-3 (see Fig. 1b). The homogeneity of the phospholipase A_2 was also confirmed by the results of isoelectric focusing, which showed that the pI value of the enzyme isolated was 5.6. From the results on the fractionation of the whole venom of *E. multisquamatus* from Sephadex G-75, revealing the localization of phospholipase A_2 in fraction II, it may be concluded that its molecular weight is between 10,000 and 25,000 daltons.

To purify the phospholipase A_2 of fraction III, characterized by a low enzymatic activity, it was chromatographed on a column of Sephadex G-25. Elution was performed with 0.05 M acetic acid solution. Three fractions were obtained: III-1, III-2, and III-3. A determination of phospholipase activity showed that this was possessed only by fraction III-1 (Fig. 1c).

On disk electrophoresis, the presence of two components in fraction III-1 was detected (see Fig. 1c), which did not permit this second phospholipase A_2 of *E. multisquamatus* venom, differing from the first by smaller molecular dimensions, to be regarded as homogeneous.

EXPERIMENTAL

The venom of the viper *Echis multisquamatus*, dried in a desiccator, was obtained from the Central Asian Zonal Zoological Combine.

Sephadexes G-25 and G-75 and the ion-exchanging SP-Sephadex G25 from Pharmacia and the marker substances dextran blue 2000, egg albumin, and cytochrome C from Serva were used.

Electrofocusing was carried out on standard polyacrylamide plates with a gradient of ampholines in the pH interval of 3.5-9.5 (LKB).

Reanal reagents were used in the electrophoretic studies.

The fractionation of the viper venom was carried out by gel filtration through Sephadex G-75. The fractions with phospholipase activity obtained after the fractionation of several portions of venom through Sephadex G-75 were combined and freeze-dried. The combined fraction was subjected to separation in a pH and molarity gradient of ammonium acetate buffer at the rate of 30 ml/h on a column (15 × 200 mm) with SP-Sephadex G-25. The sorbents were prepared in accordance with the recommendations. The issuance of protein was monitored with the aid of an ultraviolet detector of the Uvicord II type (LKB) at 280 nm, and fractions with a volume of 5 ml were collected on a chromatographic collector.

Disk electrophoresis was performed in 15% polyacrylamide gel by Reisfeld's method [6] for 150 min. The gels were stained with Amido Black 10B with subsequent clarification in 7% acetic acid for 24 h. Samples weighing 0.1-0.2 mg were introduced into the tubes.

Electrofocusing was performed on a Multiphor 2117 apparatus (LKB).

Phospholipase activities were determined by the egg yolk coagulation inhibition method [7], and the total amount of protein was measured by Lowry's method [8].

SUMMARY

1. A pure phospholipase A_2 with a molecular weight of 15 kD has been obtained from the venom of the viper *Echis multisquamatus* by gel filtration on Sephadex G-75 followed by chromatography on SP-Sephadex G-25.

2. In the gel filtration of the venom, a second phospholipase A_1, with a molecular weight below 10 kD, was isolated which was also obtained in the purified state.

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