ANALYSIS OF THE NERVE GROWTH ACTIVITIES OF PROTEIN FRACTIONS FROM THE VENOMS OF AGKISTRODON HALYS HALYS AND ECHIS MULTISQUAMATUS

L. Ya. Yukel'son, N. A. Barabanshchikova, M. G. Khafizova, R. S. Salikhov, and D. Kh. Khamidov

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The possibility has been shown of obtaining NGFs in the process of isolating coagulant proteinases and other biologically active substances from the venoms of the Agkistrodon halys halys and the Echis multisquamata. Results characterizing the NGFs of both species as basic (weakly basic) proteins with molecular masses of 30.0-40.0 kDa are presented.

Fractionation is a widely used method of overcoming the interference of the components of a snake venom, and, consequently, the detection of new substances and their isolation in the pure state with the aim of their effective use. In the overwhelming majority of cases, the method of fractionation (or a combination of several methods) is directed towards a strictly determined substance, and the other components of the venom are considered as ballast and are discarded. It is precisely such methods that we have developed previously for obtaining NGFs from Naja oxiana and E. squamatus [1, 2]. In the present work we have investigated the distribution of the NGFs in the fractions obtained in the process of isolating, in a single technological cycle, coagulant, anticoagulant, and hemorrhagic proteinases, phospholipases A_2, and other biologically active substances from the venoms of Central Asian snakes – Echis multisquamata from the family Viperidae, and Agkistrodon halys halys from the family Crotalidae. In this case our attention was turned towards the NGFs mainly by the considerable scientific interest in these substances and their commercial value as bioreagents and was connected with an endeavor to make better use of the wastes formed in the isolation of the other components of the venoms.

With the aid of chromatography on DEAE-Sephadex A-50, the whole Ag. halys halys venom was separated into 17 fractions (Fig. 1). With respect to the physicochemical properties

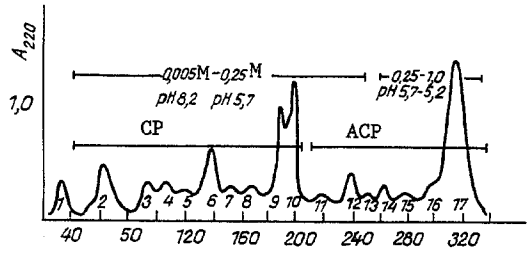

Fig. 1. Graph of the ion-exchange chromatography of the Ag. halys halys venom on DEAE-Sephadex A-50. The column (2.5 × 80.0 cm) was equilibrated with 0.005 M ammonium acetate buffer (pH 8.2). Elution program: 1) gradient of 0.005 M ammonium acetate buffer with pH 8.2-0.25 M ammonium acetate with pH 5.7; 2) gradient of 0.25 M ammonium acetate with pH 5.7-1.0 M ammonium acetate with pH 5.2. Rate of elution 30 ml/h, fraction volume 3.0 ml; 1-17) fractions combined according to the protein "peaks"; CP – coagulant part (combined fractions 1-10); ACP – anticoagulant part (combined fractions 11-17).

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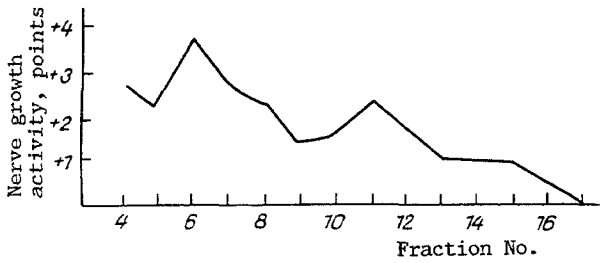

Fig. 2

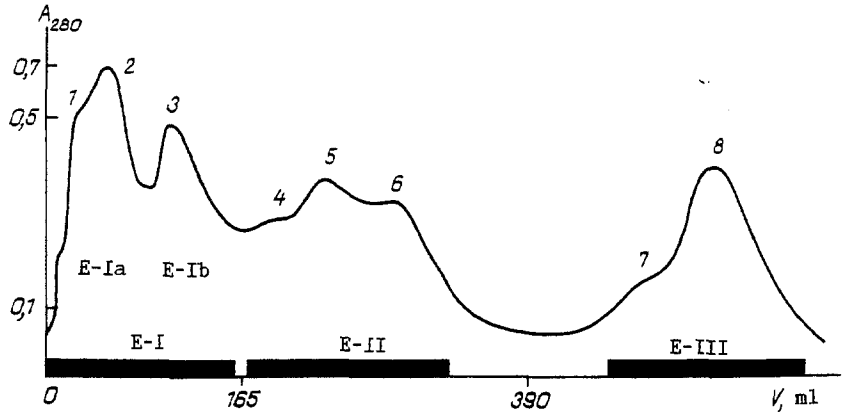

Fig. 3

Fig. 2. Distribution of nerve growth activity over the fractions obtained on the chromatography of the Ag. halys halys venom on DEAE-Sephadex A-50.

Fig. 3. Graph of the gel filtration of the viper venom on Sephadex G-7. The column (2.5 × 180 cm) was equilibrated for a day with 0.05 M ammonium acetate (pH 7.9). Elution with the equilibrating solution at the rate of 30 ml/h: 1-8) protein "peaks"; V-I–V-III) protein fractions collected preparatively.

and functional activities of the proteins predominating in the compositions of these fractions, the latter can be reduced to two groups: a coagulant part (CP) with a predominance of basic components, comprising fractions 1-10, and an anticoagulant part (ACP) comprising fractions 11-17, in which acidic proteins predominated [3]. Quantitative analysis of the levels of NGF activity in the fractions obtained (Fig. 2) revealed two maxima, corresponding to fractions 6 and 11. Isoelectric focusing showed a gradual decrease in the amounts of basic components in fractions 1-17. This permits the NGF activity of the venom to be linked with its basic components. This hypothesis is harmony with the experimental results demonstrating the basic properties (pI 9.0) of the NGFs from many snake venoms and the β-NGF from the submaxillary salivary gland of male mice [4, 5]. Thanks to the presence of specific proteinases, the fraction of basic components from the venom of Ag. halys halys has been recommended, and has undergone trials, as a diagnostic preparation for medical coagulology [6, 7]. It may be assumed that the preliminary extraction of NGFs from it will raise the efficiency of the working technology designed for the preparation of the coagulological diagnostic agent.

We have developed a special method for isolating NGFs from the venom of the Central Asian viper Echis multisquamatus [2]. Phospholipase A_2, coagulant and hemorrhagic proteinases, and a peptide carboxypeptidase inhibitor have been isolated from the same venom by other expedients [8, 9]. It is clear that the isolation of all these components of the venom in one technological chain should substantially raise the efficiency of its processing. Chromatography of the venom on molecular sieves (Fig. 3) is the most suitable method for the purification of all the above-mentioned protein factors. It appeared of interest to evaluate the localization of the NGF in the individual fractions. The investigations showed that the

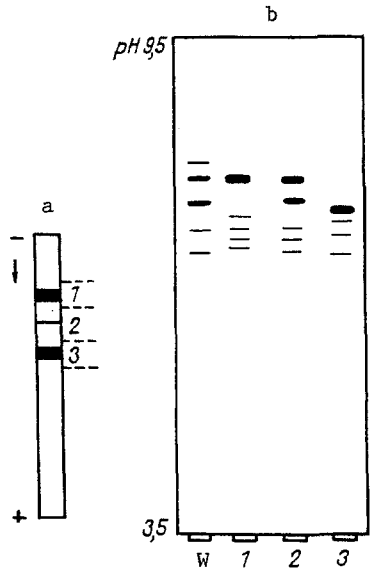

Fig. 4. Disk electrophoresis (a) and analytical isoelectric focusing (b) of the fraction obtained in the preparative isoelectric focusing of the E. multisquamatus venom: a) 7.5% PAAG, Tris-glycine buffer, pH 8.3, 4 mA/tube, 1.5 h; b) standard PAAG plates, pH interval 3.5-9.5, concentration of the sample 1.5 mg/ml, volume of the sample 20 µl, 1.5 h; W, 1-3) whole fraction obtained in the preparative isofocusing of the E. multisquamatus venom and of its fragments after electrophoresis (5a), respectively.

NGF was present fairly compactly within the first high-molecular-mass fraction (V-I). In the course of the separation it was possible to divide this fraction into two, characterized by a predominance of acidic (V-Ia) and of basic (V-Ib) components, respectively. NGF activity was found in both fractions but was substantially greater among the basic proteins (V-Ib).

The results of gel filtration, disk electrophoresis, and isoelectric focusing enable us to give the primary physicochemical characteristics of the NGF-active protein from the E. multisquamatus venom. From elution volume calculations, its molecular mass was between 30.0 and 40.0 kDa. The separation of the whole E. multisquamatus venom and of the high-molecular-mass fraction V-I on Ampholines in the pH interval of 6.0-9.5 permitted the NGF to be identified among the components with pI 7.0-8.0. Preparative isoelectric focusing of the whole venom conducted in a thin layer of Sephadex C-75 containing Ampholines (3.5-9.5) showed protein fractions focused in the pH interval of 6.0-8.5, and these were separated from the Ampholines (yield 18% on the weight of the venom) and subjected to column gel filtration on Sephadex C-50 or C-75; molecular mass calculations for the NGF-active components gave the same value of 30.0-40.0 kDa. It was impossible to achieve further purification of the NGF by gel filtration. The focused protein material was separated into three fractions by electrophoresis (Fig. 4a), and NGF activity was determined in fraction 1, possessing the lowest mobility. Isoelectric focusing revealed in fraction 1 the presence of a single NGF-active protein and a very small amount (less than 5%) of minor components as impurities (Fig. 4b). In this process, the NGF protein was focused in the pH interval of 7.8-8.2, and its activity amounted to 10^5 BU/mg of protein. These preliminary results characterizing the molecular parameters of the NGF protein were later confirmed by its isolation in the pure form [2].

A knowledge of the physicochemical properties of the NGF protein of the E. multisquamatus venom will, of course, facilitate the development of a method oriented exclusively to the isolation of this substance [2]. It may be hoped that they will also be useful in the creation of a unitary scheme, for the isolation in the purified state of a number of the biologically active substances that have been detected in the E. multisquamatus venom. In this connection, attention must be directed to the fact that, in the process of gel-filtering the NGF, coagulant, hemorrhagic, and other proteinases can be separated, as components of a high-

molecular-mass fraction, from the phospholipase A_2 and peptide proteinase inhibitors, and this fraction may be recommended as a source of NGF and proteinases.

EXPERIMENTAL

We used a batch of $CaCl_2$-dried venoms of Central Asian snakes – Ag. halys halys Sch. and Echis multisquamatus Ch., which were purchased from the Uzbek Zoological Combine, Sephadexes of various brands and types from Pharmacia (Sweden), and kits for disk electrophoresis and for isoelectric focusing from Reanal (Hungary) and LKB (Sweden); the other reagents were of foreign or domestic production with the grades KhCh ["chemically pure"] or ChDA ["pure for analysis"]. For cultivating chick embryo explants we used Eagle's medium, medium 199, Hanks' solution, bovine serum albumin (of domestic production), and a home-prepared solution of collagen. Before use in the biotest, the solutions were sterilized by passage through a bacterial filter with a pore size of 0.2 μm.

The chromatographic and electrophoretic experiments were conducted in accordance with the recommendations of the manufacturers of the corresponding instruments or reagent kits. The NGF effect was judged from the formation of a halo in the form of a fibrillar frame around the explants. The density of the halo was evaluated on a points scale [9]. The NGF activity was calculated to biological units (BU): as one BU we took that amount of protein (ng) that caused the formation of a halo having an estimated score of three points (+3).

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