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PURIFICATION OF NERVE GROWTH FACTOR FROM THE VENOM OF THE CENTRAL ASIAN COBRA *Naja oxiana*

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A highly purified electrophoreticaly homogeneous protein with a NGF activity of $10 \cdot 10^5$ BU*/mg of protein have been isolated from the venom of the Central Asian cobra by gel-filtration and ion-exchange chromatography followed by preparative isolectric focusing in a thin layer of Sephadex. It has been shown that the NGF isolated is characterized by a molecular weight in the range of 20-30 kD and a pI value of about 7.0.

Nerve growth factor (NGF) is a protein which is known for its capacity for stimulating the growth of nerve fibers with the formation of a so-called halo of axons in cultivated spinal ganglia of chick embryos. Various effects of NGF have been described in the literature and an analysis of these shows its importance in the development of the nervous system, especially the sympathetic innervation [1, 2]. As a molecular factor of development, NGF presents considerable interest, particularly for neurobiology and this explains the numerous attempts to obtain in the purified state from various tissues and biological fluids [2-4]. Snake venom is considered to be a rich source of NGF and, in the relevant literature [3], detailed information is given which characterizes methods of isolating snake venon NGF and its molecular properties. This information indicates the existence of NGFs differing in structure and properties from different sources. In this connection, attention must be directed to the absence of sufficient information relative to NGF in the venoms of Central Asian snakes. Only recently has there been publication of investigations characterizing the level of activity of NGF in these venoms and attempts to obtain it in the purified state [5-7]. The present paper gives the results of work on the purification of NGF from the venom of the Central Asian cobra *Naja oxiana* Eichw.

In the first stage of purification of the venon of the Central Asian cobra was fractionated by gel-filtration on Sephadex under the conditions developed previously in connection with the isolation of pure phospholipases A_2, and also neuro- and cytotoxins from this venom [8-12]. A standard separation of the venom into three fractions [I-III, Fig. 1) was achieved, but small changes in the parameters of the gel-containing column and the eluent, and also in the basic technique of recording the optical density of the eluate, which was performed continuously with the aid of a Uvicord II instrument (LKB, Sweden) permitted the identification of six protein fractions 1-6. Biological testing showed that the main zone of NGF activity corresponded to the area of peak 4, which contained components with molecular weights in the range of 20-30 kD. Slight NGF activity was also detected in fractions 3 and 5. The results of isolectric focusing on PAAG plates with Ampholines in the pH range of 3.5-9.5 showed the presence in fractions 4 to 20 of protein components with pI values between 6.0 and 9.5. In the light of the predominant presence in these fractions of basic components, the further purification of the NGF was performed by chromatography on CM-cellulose.

*BU signifies biological units – Publisher.

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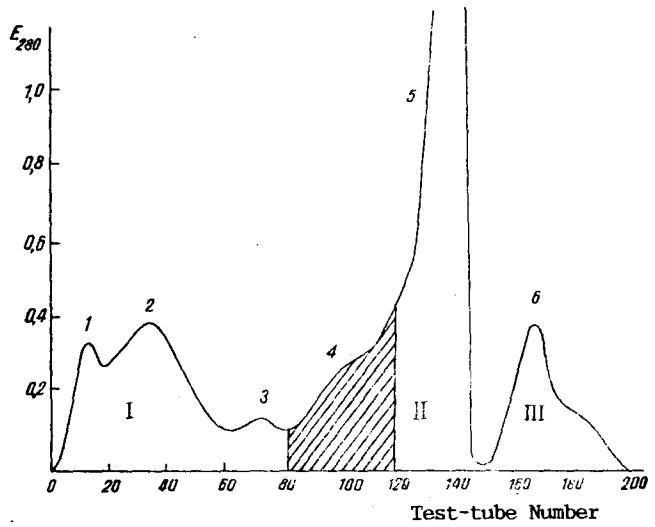

Fig. 1. Gel-filtration of 500 mg of the venom of the Central Asian cobra on a column of Sephadex G-100 (2.6 × 90 cm): eluent – 1% acetic acid, rate of elution 30 ml/h. 1-6) protein "peaks" in the order of their elution from the column; I, II, III) fractions combined according to the protein "peaks." The zone with NGF activity is hatched.

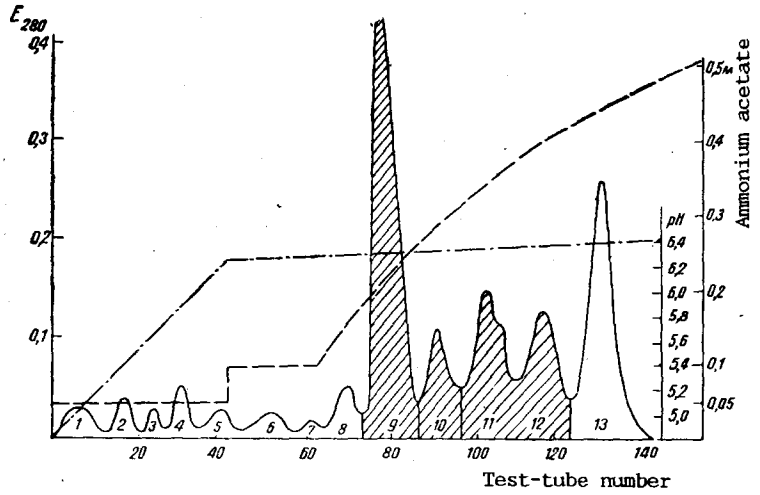

Fig. 2. Chromatography of 200 mg of fraction 4 of cobra venom on a column of CM-cellulose (1.9 × 5.5 cm): the experimental conditions are given in the text; rate of elution 50 ml/h; 1-13) fractions combined according to the protein "peaks." The zone with NGF activity is hatched.

The conditions for the absorption and chromatography of the main components of the venon of the Central Asian cobra on CM-cellulose had been developed previously [9-12]. A column containing CM-cellulose was equilibrated with 0.05 M ammonium acetate buffer (pH 4.8) and fraction 4, previously freeze-dried and dissolved in the equilibrating solution, was adsorbed on it. The following elution program was used: 1) linear pH gradient from 4.8 to 6.2 of 0.05 M ammonium acetate buffer; 2) 0. 1 M solution of ammonium acetate (pH 6.2); 3) 0.2 M solution of ammonium acetate (pH 6.2); 4) linear concentration gradient of ammonium acetate solution from 0.2 to 0.5 M (pH 6.4); 5) 1.0 M solution of ammonium acetate (pH 6.5); 6) 2.0 M solution of ammonium acetate (pH 6.5). Under these conditions it was possible to separate frac-

tion 4 into 13 components (Fig. 2). Below we give information characterizing the nerve-growth activity of the fractions obtained on the well-known scale from 0 to +5 [12]:

Fraction	Concentration, μg/ml	NGF activity
1—8	20	0
9	20	+5
	10	+5
	7	+5
	4	+3
10	20	+5
11	20	+4
	10	+3
	7	+3
	4	+2
12	20	+4
13	20	+2

The results obtained show a distribution of the NGF activity among four of the components of fraction 4. The greatest nerve-growth activity was found in fraction 9, the weight of which was greater than that of the other components of fraction 4. More accurate measurements of the NGF activity of this fraction connected with a determination of the lowest concentration of it causing a standard response in the biotest enables it to be calculated as 8×10^5 BU/mg of protein.

Disk electrophoresis showed the presence in fraction 9 of two components, of which one migrated rapidly to the anode in an acidic buffer medium. However, analytical isolectric focusing in the pH range of 3.5-9.5 showed the presence in fraction 9 of a considerably larger number of individual proteins with pI values between 6.0 and 8.0. Assuming that this heterogeneity may be due to the glycoprotein nature of the NGF [3], we treated fraction 9 with neuraminidase and subjected it to electrophoresis. In another variant of the experiments, electrophoretograms of fraction 9 were used for detecting sugars with the aid of Schiff/periodic acid reaction. The results of the experiments showed the absence of sugars in the preparations of NGF from the venom of the Central Asian cobra, which is harmony with the analogous information relative to the structure of the NGFs isolated from the venoms of other elapid snakes [14].

The further purification of the NGF was performed by the preparative isolectric focusing of fraction 9 in a thin layer of Sephadex G-75 Superfine using Ampholines in the narrow pH range of 6.0-8.0. In this way, fraction 9 was separated into three components, I_1, I_2, and I_3 (Fig. 3, a) one of which (I_2) was characterized by electrophoretic homogeneity (Fig. 3, b) and a high nerve-growth activity, of $10 \cdot 10^5$ BU/mg of protein.

The facts given show that with the aid of gel-filtration of cobra venom followed by chromatography of the active fraction on a cation-exchange resin it is in fact possible to achieve some purification of the NGF but it is not possible to obtain it in the pure form in this manner. Thus, our results do not confirm those relating to the NGF from the venom of the Indian cobra Naja naja which under almost identical conditions was isolated from the cobra in the individual state but they do agree with investigations of the venom of the Formosan cobra Naja naja atra, where gel filtration and ion-exchange chromatography led to the isolation of a material with nerve-growth activity which was likewise heterogeneous in its physicochemical properties [15]. A similar two-stage procedure consisting of gel filtration and chromatography did not lead to success, either, in the case of the venoms of other snakes both of the elapid and other families [3, 6, 14]. The inclusion of an additional stage of purification – for example, gel-filtration on Sepharose [15] or preparative isoelectric focusing, as we have done – permits an electrophoretically homogeneous NGF to be obtained which it is possible to characterize in preliminary fashion as a result of experiments on its isolation.

The results of the experiments on the gel-filtration of the cobra venom showed the position of the NGF among the components with molecular weights of 20-30 kD. This in harmony with literature information [3, 6, 14, 15] establishing molecular weight of 20-35 kD for the NGF from various venoms. With respect to their isoelectric points, the NGFs from different venoms are extremely different from one another [14]. However, the NGFs from the venoms of elapid snakes have close pI values: 6.75 for that from the venom of the Indian cobra [3] and 7.02 for that from the venom of the Formosam cobra [15]. We found a similar pI value of 7.0 for the component with NGF activity isolated in the electrophoretically homogeneous state

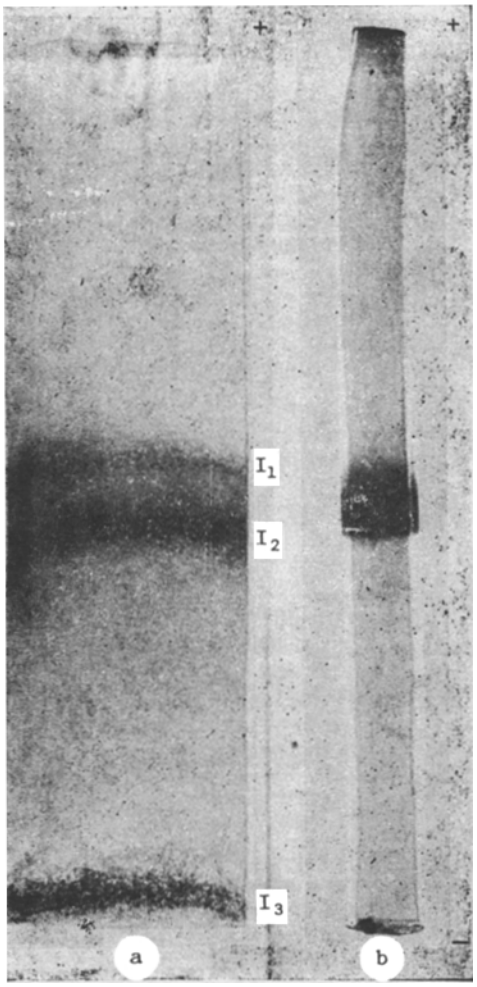

Fig. 3. Preparative isoelectric focusing of 35 mg of the NGF fraction (a) and disk electrophoresis of fraction I_2 using 7.5% PAAG and an electrode buffer solution with pH 4.0 (b).

with the aid of isoelectric focusing. Literature information [14] also confirmed the absence of reactions for sugars in preparations of the NGFs from elapid venoms, and these facts in combination may indicate their great structural similarity. It is assumed [3, 14, 15] that the molecules of the NGFs from elapid venoms are organized in the form of dimers with identical subunits having a molecular weight of 10-12 kD and therefore the microheterogeneity of NGF preparations is probably due to the simultaneous presence of dimers and monomers differing in their pI values which can be differentiated with the aid of isoelectric focusing. In contrast to these, the NGFs from the venoms of other snakes [6. 14] are glycoproteins and have a more complex structural organization, which can explain the heterogeneity of their purified preparations observed in experiments on the electrophoresis or isoelectric focusing of these preparations.

EXPERIMENTAL

The venom of the Central Asian cobra was obtained from the Laboratory for the Ecology of Poisonous Snakes of the Institute of Zoology and Parasitology of the Academy of Sciences of the Uzbek SSR.

Samples of venom dried over calcium chloride (1982-1983 collection) were used. The venom was fractionated on a column of Sephadex G-100 (26 × 900 mm) followed by the ion-exchange chromatography of the active fraction on CM-cellulose (19 × 550 mm column).

The collection of the eluate (volume 4-5 ml) and the recording of the fractions was performed on an LKB automatic device. The fractions were combined according to the protein peaks, concentrated in a rotary evaporator, desalted on a column of Sephadex G-25, reconcentrated and freeze-dried.

Preparative isoelectric focusing was performed in a thin layer of Sephadex G-75 Superfine with 2% of Ampholines in the pH range from 6.0 to 8.0 for 10 h at a constant power of 8 W. The components of the buffer mixture were: cathode buffer – 1 M NaOH; anode buffer – 1 M H_3PO_4.

The gels were divided into zones 1 cm wide. The protein was eluted from the zones and was desalted on Sephadex G-50.

Nerve-growth activities were estimated from the stimulating action of the individual fractions on the growth of axons from the spinal ganglia of 7- to 10-day chick embryos. Cultivation was carried out by the flying coverslip method [16] in a nutrient medium containing Eagel's medium, Hanks' solution, and bovine serum albumin in a ratio of 50:50:12.

SUMMARY

1. A highly purified electrophoretically homogeneous protein with an NGF activity of $10 \cdot 10^5$ BU/mg of protein has been obtained from the venom of the Central Asian cobra by gel-filtration, ion-exchange chromatography, and preparative isoelectric focusing in a thin layer of Sephadex.

2. The NGF isolated has a molecular weight in the range of 20-30 kD and a pI value of $\sim$7.0.

3. The NGF isolated gives no positive reaction for sugars and its isoelectric migration does not change after treatment eith neuraminidase.

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