Journal of Chromatography, 281 (1983) 389–392
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,203

Note

Separation of cardiotoxins (cytotoxins) from the venoms of *Naja naja* and *Naja naja atra* by reversed-phase high-performance liquid chromatography

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(Received August 5th, 1983)

It is known that the venoms of snakes belonging to the family *Elapidae* contain a large amount of membrane-active basic proteins, cardiotoxins (cytotoxins) (CTXs), which cause irreversible depolarization of the cell membrane[1]. These proteins also cause systolic arrest of the heart[1] and show preferential cytotoxicity toward Yoshida sarcoma and ascites hepatoma cells[2]. Cardiotoxins are single polypeptides consisting of 60 amino acid residues with four intramolecular disulphide linkages[3].

We have previously isolated these proteins from the venoms of Indian (*Naja naja*)[4–6] and Formosan (*Naja naja atra*)[7] cobras, and designated them as cytotoxins I, IIa and II, and cardiotoxin analogues I, II, III (cardiotoxin) and IV, respectively, according to their sequential elution from a CM-cellulose column. Studies of the amino acid sequences revealed that these proteins are highly homologous in their primary structures[4–9]. Although these proteins have been purified by means of the combination of gel-filtration and CM-cellulose column chromatography, complete separation could not be attained. It seems important to check the purity of a component in order to investigate in detail the mechanism of action of the toxin on the plasma membrane, because it is conceivable that the effects of these components on the membrane differ depending on the slight differences in their structures. It was, however, difficult to use polyacrylamide gel electrophoresis for checking the purity, because of their extremely positive charges and similar molecular weights.

This paper deals with the complete separation of these cardiotoxins (cytotoxins) by means of reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Cytotoxins I, IIa and II, and cardiotoxin analogues I, II, III and IV were purified as previously described from the venoms of *Naja naja*[4–6] and *Naja naja atra*[7], respectively, which were purchased from Sigma, St. Louis, MO, U.S.A. Trifluoroacetic acid (TFA) and acetonitrile (>99.5%) of extra pure grade and chromatography grade, respectively, were obtained from Nakarai Chemicals, Kyoto, Japan.

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Apparatus

The HPLC system used was a Shimadzu Model LC-3A liquid chromatograph with a linear gradient maker (Model GRE-2B). A stainless-steel column (250 × 4.6 mm I.D.) was packed with Nucleosil 5CN (particle size 5 μm, Macherey-Nagel, Düren, F.R.G.) suspended in carbon tetrachloride by the slurry packing method (packing pressure, 400 kg/cm^2). Absorbance at 230 nm was monitored with a Shimadzu SPD-1 spectrophotometer and recorded with a Shimadzu C-R1A recording integrator.

Chromatographic conditions

Solvents A and B consisted of 0.1% TFA–acetonitrile (5:1 for solvent A, and 1:1 for solvent B, v/v). Both solvents were degassed for a few minutes with a water aspirator prior to use. The linear gradient was made by adding solvent B to solvent A at a rate of 0.5%/min until 25% solvent B was reached. The column was recycled with solvent A for 15 min. The flow-rate of the mobile phase was 0.7 ml/min, which produced a column pressure of *ca.* 140 kg/cm^2. The column temperature was controlled at 38°C. A sample of 20–100 μg of protein for each component was injected.

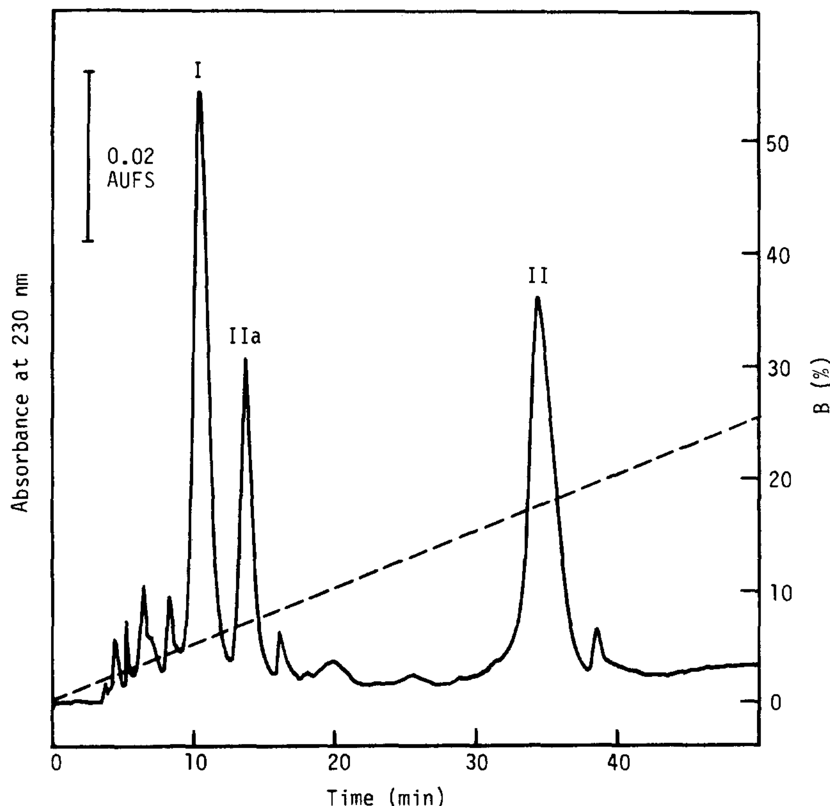

Fig. 1. Separation of cytotoxins, I, IIa and II from the venom of *Naja naja* on a Nucleosil 5CN column (250 × 4.6 mm I.D.). The initial solvent A was 0.1% TFA–acetonitrile (5:1) and the secondary solvent B was 0.1% TFA–acetonitrile (1:1). The flow-rate was 0.7 ml/min and the column temperature was 38°C. The amounts of CTXs, I, IIa and II injected were 122, 30 and 120 μg, respectively.

RESULTS AND DISCUSSION

Cytotoxins I, IIa and II from the venom of *Naja naja,* which were purified by conventional CM-cellulose column chromatography, were dissolved in solvent A and analysed on a Nucleosil 5CN column. Fig. 1 shows the elution profile of these toxins: the three components were completely separated within *ca.* 40 min. The concentration of acetonitrile at 40 min was 23.3%. More rapid analysis (*ca.* 25 min) was also possible when acetonitrile was used as solvent B (data not shown).

Similarly, four cardiotoxin analogues from the venom of *Naja naja atra* were clearly separated on a Nucleosil 5CN column (Fig. 2). These toxins were eluted from the column in the order of CTX analogues I, IV, II and III.

Generally, in conventional CM-cellulose column chromatography, cardiotoxins (cytotoxins) were eluted in broad peaks, and clear separation was very difficult. For example, a minor component, cytotoxin IIa, was eluted as a shoulder of the peak of cytotoxin II, and was much contaminated by cytotoxins I and II. In the case of the venom from *Naja naja atra,* CTX analogues II, III and IV were eluted with considerable overlapping, and only CTX analogue I was separated completely from

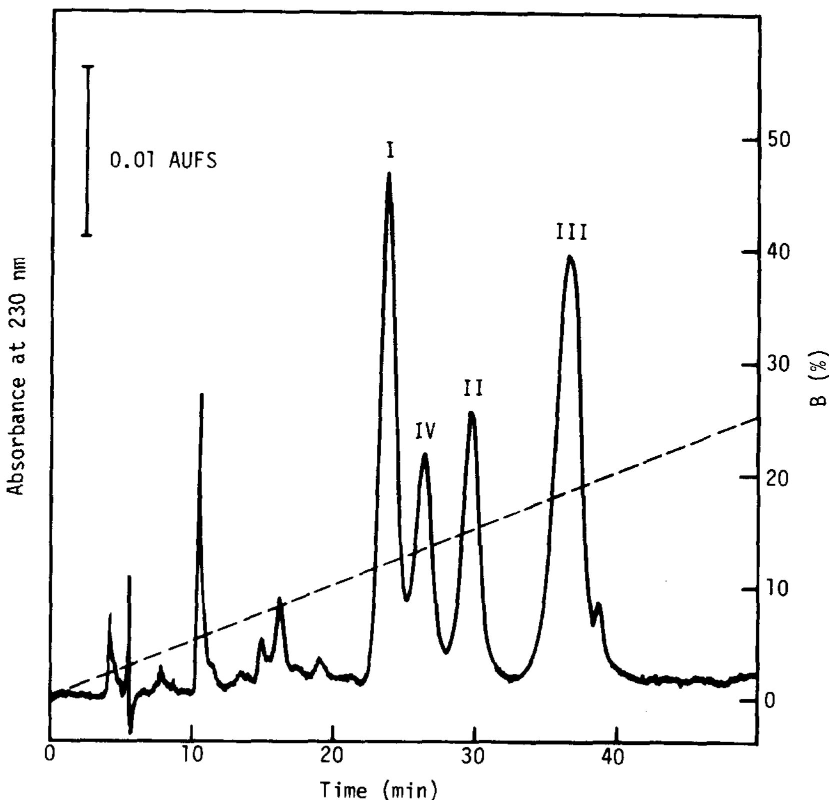

Fig. 2. Separation of cardiotoxin analogues I, II, III and IV from the venom of *Naja naja atra* on a Nucleosil 5CN column (250 × 4.6 mm I.D.). The chromatographic conditions were the same as those in Fig. 1. The amounts of CTX analogues I, II, III and IV injected were 53, 25, 55 and 35 μg, respectively.

these components. These problems were easily solved by using this HPLC system (Fig. 2).

Recently, Wu *et al.*[10] reported the separation of four cardiotoxins of *Naja naja atra* by means of reversed-phase HPLC. They compared several reversed-phase supports, and found the best separation on a μBondapak-phenyl column, but inadequate separation on a μBondapak-CN column. In addition, there was some difficulty in separating CTX analogues II and IV, because these two proteins differ only in one amino acid residue, at position 1[7]; Leu in CTX analogue II and Arg in CTX analogue IV. Comparing these results with those presented here, the present system exhibits excellent resolution for these highly homologous components.

When compared with conventional polyacrylamide disc gel electrophoresis, this method has many advantages such as high resolution, rapid analysis, and easy recovery of the material. In the present study, we used a mixture of 0.1% TFA and acetonitrile as the mobile phase. Because these solvents can be removed by lyophilysation, this method may also be useful in the purification of these toxins in larger amounts when a preparative column is used.

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