ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 1163–1167

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Fast protein liquid chromatography for the purification of animal venoms[1]

Tom Debont[a,*], Paul Daenens[a], Jan Tytgat[a,b]

[a]*Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, University of Leuven, E. Van Evenstraat 4, B-3000 Leuven, Belgium*

[b]*Laboratory of Physiology, Faculty of Medicine, University of Leuven, Campus Gasthuisberg, O & N, B-3000 Leuven, Belgium*

Received for review 13 September 1995; revised manuscript received 4 December 1995

Abstract

In this paper a methodology is presented for the purification of toxins acting on neuronal potassium channels. These neurotoxins are ideal tools for studying the physiological functioning of ion channels, for classifying them and for exploring them in several neuronal regions. Separation procedures leading to the identification of neurotoxins almost always include gel filtration chromatography as well as other chromatographic techniques such as ion exchange and reversed phase chromatography. The use of a Superdex 30 prep grade gel filtration column, which has the advantage of superior resolution in comparison with traditionally used gel filtration columns such as a Sephadex G-50 column, is reported here. The biological assay incorporates the use of *Xenopus laevis* oocytes, which express potassium channels. Screening of the collected venom fractions was performed by means of the whole-cell voltage clamp technique. The combination of these techniques represents a fast and efficient identification procedure in the search for new and selective neurotoxins for cloned channels and receptors.

Keywords: Natural toxins; Oocyte; Potassium channel; Purification; Snake venom; Voltage clamp

1. Introduction

Snake venoms, like other venoms from marine and terrestial animals, consist of a very complex mixture of components [1]. Among these components are toxins which are specifically directed against the nervous system. These neurotoxins are molecules that often affect only one function of the nervous system and thereby one key receptor molecule. Because of this specificity they are ideal tools for the study of the important receptor molecules in the nervous system and for the exploration of basic mechanisms of nerve functioning [2].

In this paper, a combination of chromatographic techniques with a biological assay for the purification of neurotoxins from the venom of the green mamba snake, *Dendroaspis angusticeps*, is described. The Fast Protein Liquid Chromatography (FPLC)® System from Pharmacia LKB Bio-

* Corresponding author. Tel.: (+32) 16-32-34-07; fax; (+32) 16-32-34-05.

[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01747-5

technology Inc. (Sweden) is used, which is especially designed for the separation of biomolecules. This system has the advantage that it can be operated with different types of columns (gel filtration, ion exchange and reversed phase chromatography columns), which allow preparative separations, in contrast to most HPLC columns, and sometimes also offer a better resolution. This is especially true for the gel filtration column used in this separation protocol, a Superdex 30 prep grade HiLoad 16/60. In combination with the used separation techniques (gel filtration, ion exchange and reversed phase), a fast screening method for measuring the biological activity of the sample components was developed. The biological assay included the use of voltage-dependent potassium channels which were studied by means of the whole-cell voltage clamp technique [3]. This technique was performed on *Xenopus laevis* oocytes which were injected with mRNA coding for a neuronal voltage-dependent potassium channel. The combination of the aforementioned techniques represents a fast and efficient identification procedure in the search for new and selective neurotoxins for cloned channels and receptors.

2. Materials and methods

2.1. Venom fractionation

Crude venom from the green mamba, *Dendroaspis angusticeps*, was obtained from Sigma Chemical Co. (USA). The lyophilized venom of *Dendroaspis* was dissolved in 100 mM ammonium acetate (Merck, Germany) buffer, pH 6, at a concentration of 100 mg ml^{-1}. 500 μl of the venom suspension was then filtered through a 0.2 μm pore filter (Acrodisc 13 filter, Gelman Sciences, USA) and loaded onto a Superdex 30 prep grade HiLoad 16/60 gel filtration column (Pharmacia LKB Biotechnology Inc., Sweden) equilibrated with 100 mM ammonium acetate buffer, pH 6. The material was eluted from the column at a flow rate of 0.2 ml min^{-1} overnight, driven by an FPLC® System (Pharmacia) and controlled by the FPLC director® software (version 1.03) on a Compaq Deskpro XE 466 computer. Absorbance of the eluate was monitored at 280 nm. Fractions were collected automatically (fraction collector FRAC-200, Pharmacia) and each contained 4 ml. Fraction 16 was further purified on a Mono S HR 5/5 cation exchange column, fraction 16 was eluted through a Sephadex G-25 (PD-10 column, Pharmacia) packed column to obtain a buffer exchange for sodium phosphate. Elution of the loaded sample (2.5 ml) on the Mono S was performed by using a linear gradient from 15–65% of a 1 M NaCl solution (in 50 mM sodium phosphate, pH 6.8) for 60 min. The flow rate was 1 ml min^{-1} and the absorbance was measured at 280 nm. Fraction 5 was desalted by using another PD-10 column and then freeze-dried in order to re-dissolve the material in a 0.1% trifluoro acetic acid solution (1000 μl) in distilled water. 200 μl of this sample was loaded onto a PepRPC HR 5/5 C_2/C_{18} reversed phase column (Pharmacia) equilibrated with 0.1% trifluoro acetic acid (TFA) in distilled water. Separation was performed by using a linear gradient from 0–100% acetonitrile (supplemented with 0.1% TFA) for 100 min. The flow rate used was 0.2 ml min^{-1} and the absorbance was measured at 280 nm.

2.2. Screening procedure

The cDNA encoding the neuronal *RCK1* K channel [4] was subcloned in a 3 kb high-expression vector, called pGEMHE, based on pGEM-3Z (Promega, USA) containing 5′ and 3′ non-translated sequences of a *Xenopus* β-globin gene flanking the channel cDNA [5]. For in-vitro transcription, the plasmid was first linearized with *Pst*I (New England Biolabs, USA) 3′ to the 3′ non-translated β-globin sequence and then transcribed with the "Riboprobe Gemini System" (Promega, USA) using T7 RNA polymerase and a cap analogue diguanosine triphosphate. Stage V–VI *Xenopus laevis* oocytes were isolated by partial ovariectomy under anaesthesia (tricaine, 1 g l^{-1}). Anaesthetized animals were then kept on ice during dissection. The oocytes were defolliculated by treatment with 2 mg ml^{-1} collagenase (Boehringer, Germany) in zero calcium ND-96 solution, containing (in mM): 96 NaCl, 2 KCl, 1.8

$CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.5, supplemented with 50 mg ml^{-1} gentamycin sulphate (for incubation only). Between 2 and 24 h after defolliculation, oocytes were injected with 50 nl of 1–100 ng μl^{-1} mRNA. The oocytes were then incubated in ND-96 solution at 18°C for 1–4 days. Whole-cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique (GeneClamp 500, Axon Instruments, USA). Resistances of voltage and current electrodes were kept as low as possible (voltage electrode: 0.1–0.5 MΩ; current electrode: 0.1 MΩ) and were filled with 3 M KCl or 1 M K_3citrate plus 10 mM KCl. Current records were sampled at 1000 μs intervals and filtered at 500 Hz, using a four-pole low-pass Bessel filter. To eliminate the effect of the voltage drop across the bath grounding electrode, the bath potential was actively controlled, as measured near the outside surface of the oocyte by means of a two-electode virtual-ground circuit (also called "bath clamp"). Voltage records were carefully monitored on an oscilloscope (Hambeg, Germany) to ensure fast and proper clamping, and only adequately clamped oocytes were qualified for storage and off-line analysis on an IBM-compatible 90 MHz Pentium PC. Linear components of capacity and leak currents were not subracted. Capacitative currents did not interfere with steady-state measurements of *RCK1* currents, and leak current amplitudes were only in the range of $\approx 1\%$ of the amplitude of time- and voltage-dependent *RCK1* currents. In non-injected or H_2O-injected oocytes ($n = 20$), endogenous currents observed in the tested voltage range amounted only to $\approx 1\%$ of the amplitude of *RCK1* currents. All electrophysiological experiments were performed at room temperature (19–23°C). Upon transferring the oocytes to the recording chamber, the ND-96 medium bathing the oocytes in the incubation dishes was replaced with the 100 mM ammonium acetate buffer solution (pH 6), supplemented with (in mM) 6 NaCl, 2 KCl, 1.8 $CaCl_2$, and 1 $MgCl_2$ (for gel filtration screening). Collected gel filtration fractions which were selected for screening were also supplemented with (in mM) 6 NaCl, 2 KCl, 1.8 $CaCl_2$, and 1 $MgCl_2$. Fractions from ion exchange and reversed phase chromatograms were screened in the ND-96 medium (pH 7.5). Phenotypes of voltage-dependent K currents evoked by depolarizing test pulses were identical in the two buffered solutions (Hepes buffer in ND-96 vs. ammonium acetate buffer). 50 μl of all different fractions was added directly to the recording chamber (with a volume of 500 μl).

3. Results and discussion

Fig. 1 displays the gel filtration chromatogram of *Dendroaspis angusticeps* venom which was obtained by using a Superdex 30 prep grade HiLoad 16/60 FPLC column. The suspended and filtered material (50 mg per 500 μl) was injected and then applied onto the column. Next, the material was eluted over a period of 800 min at a flow rate of 0.2 ml min^{-1} and collected in 40 individual fractions. During the first 200 min, no elution of material was observed. However, between 200 and 425 min, the bulk of the venom was eluted. This bulk fraction was characterized by many peaks with one sharp peak corresponding to the highest absorbance. Around 550–600 min, two minor absorbances were registered and, finally, a massive peak at around 720 min was observed. All

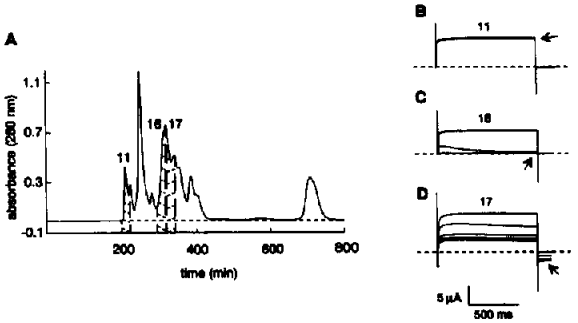

Fig. 1. (A) Gel filtration chromatogram of *Dendroaspis angusticeps* venom on a Superdex 30 prep grade HiLoad 16/60 column in 100 mM ammonium acetate, pH 6. The venom was dissolved in 500 μl 100 mM ammonium acetate, pH 6. The sample was filtered and applied to the column. All fractions were screened for their biological effect, with fractions 11, 16 and 17 being representative examples. Fraction 11 (B) showed no effect at all. Fraction 16 gave rise to a selective inhibition of the (*RCK1*) potassium current (C), whereas fraction 17 (D) also affected the holding current.

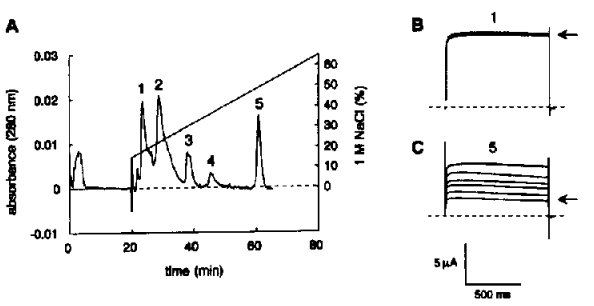

Fig. 2. (A) Ion exchange chromatogram of fraction 16 from the gel filtration on a Mono S HR 5/5 cation exchange column equilibrated with 50 mM sodium phosphate, pH 6.8. Elution of the loaded sample (2.5 ml) was performed by using a linear gradient from 15–65% of a 1 M NaCl solution (in 50 mM sodium phosphate, pH 6.8) for 60 min. Separation resulted in five fractions with only fraction five having a biological inhibitory effect (C). (B) shows the lack of effect of fraction one on the potassium current.

fractions with UV-absorbing material were then screened for their biological potential for inhibiting a neuronal-cloned voltage-gated *RCK1* potassium channel. Whole-cell voltage clamp experiments were performed on *Xenopus laevis* oocytes which expressed *RCK1* potassium currents, in order to define possible different effects of the isolated fractions on the potassium currents. The protocol used in this screening consisted of clamping the oocyte from a holding potential (V_{hold}) of -90 mV to a test potential (V_{test}) of 0 mV for 1

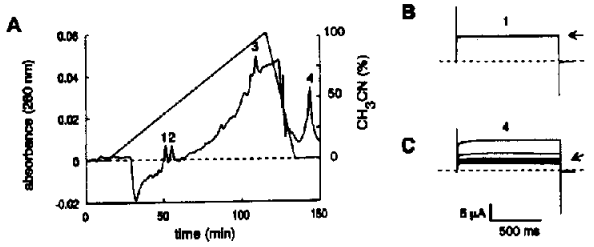

Fig. 3. (A) Reversed phase chromatogram of fraction five of ion exchange performed on a PepRPC HR 5/5 C_2/C_{18} reversed phase column equilibrated with 0.1% trifluoro acetic acid in distilled water. Separation was performed by using a linear gradient form 0–100% of 0.1% trifluoro acetic acid in acetonitrile for 100 min. This separation yielded four fractions with a lack of effect of fraction one (B) and a selective effect of fraction four (C).

s. This protocol was repeated seven times at a frequency of 0.2 Hz. Depolarization resulted in an outward, fast-activating and non-inactivating "delayed rectifying-type" potassium current (I_K). Extracellular application of the different venom fractions induced three kinds of effect on the potassium current, i.e. no effect, a selective inhibitory effect on I_K and a non-selective effect on the membrane conductance. Representative fractions, which illustrate these effects, were chosen and consist of fractions 11, 16 and 17. Applications of fraction 11 did not influence the potassium current (Fig. 1B), whereas fraction 16 induced a complete and selective inhibition of I_K during wash-in (Fig. 1C). In contrast, wash-in of fraction 17 showed effects on the current both at V_{hold} and V_{test} (Fig. 1D). By screening of fraction 17, the oocytes always became "leaky" and proper voltage control was often impossible. Fraction 16 was then further studied and used in the purification protocol because of its selective effect on the potassium current under voltage clamping.

The purification of toxins from animal venoms, performed on a FPLC® (Fast Protein Liquid Chromatography) System, is a patented technique by Pharmacia. Although the manufacturer's definition states "fast" chromatography, ideal gel filtration experiments still took 800 min, which is clearly not fast. Therefore, the speed of the system should not be taken too literally.

Gel filtration chromatograms obtained with the Superdex 30 prep grade HiLoad 16/60 column, being the first step in the purification process of venoms, lead to a separation with much better resolution compared to the traditionally used gel filtration media packed columns. In the literature, Sephadex G-50 packed gel filtration columns are omnipresent in gel filtration chromatography. However, chromatograms obtained with this type of column are characterized by the presence of broad absorbances without any detectable isolated sharp peaks [6,7]. In contrast, in the chromatograms obtained in this work several peaks can be detected which are much narrower in comparison to the traditional chromatograms.

The use of oocytes in the biological assay constitutes a heterologous expression system for the

injected mRNA coding for potassium channels. This system has many advantages: (i) the oocyte has only a few endogeneous channels, which usually carry only a very small fraction of the total current expressed; (ii) oocytes faithfully express foreign RNA that can easily be injected; (iii) the cells are quite hard and can tolerate repeated impalements of microelectrodes; and (iv) hundred of viable cells can be isolated from a single *Xenopus laevis* frog.

The second step in the purification consisted of separation on a cation exchange column, i.e. a Mono S HR 5/5 column (Pharmacia). The separation of the 2.5 ml loaded sample resulted in a chromatogram with five peaks (Fig. 2). Only the last peak showed a nearly symmetrical peak pattern combined with a biological effect (Fig. 2C). All the other fractions (1–4) displayed no inhibitory effect on the potassium current, with fraction 1 shown in Fig. 2B.

The final step in the purification process was then applying fraction five of the ion exchange chromatogram onto a PepRPC HR 5/5 reversed phase column packed with porous silica coupled to C_2/C_{18} chains (Pharmacia). The separation of the 200 μl loaded sample resulted in a chromatogram with four peaks (Fig. 3). Only the fourth peak, which eluted after gradient application, gave rise to an inhibition of the potassium current (Fig. 3C). Elution of this component after the gradient may be because of its solubility. During gradient formation the component might become insoluble in the acetonitrile phase. When returning to basic conditions, the fraction redissolves and elutes from the column. The other fractions had no effect on the potassium current, as illustrated for fraction one in Fig. 2B.

Acknowledgements

J.T. is a research associate of the N.F.W.O. (Belgium).

References

[1] B. Rees and A. Bilwes, Chem. Res. Toxicol., 6 (1993) 385–406.
[2] F. Hucho, Angew. Chem., Int. Ed. Engl., 34 (1995) 39–50.
[3] B. Hille, in Ionic Channels of Excitable Membranes, 2nd edn., Sinauer Associates Inc., Sunderland, MA, 1991, pp. 23–58.
[4] A. Baumann, A. Grupe, A. Ackermann and O. Pongs, EMBO J., 7 (1988) 2457–2463.
[5] E. Liman, J. Tytgat and P. Hess, Neuron, 9 (1992) 861–871.
[6] C. Benishin, R. Sorensen, W. Brown, B. Kreuger and M. Blaustein, Mol. Pharmacol., 34 (1988) 152–159.
[7] A.L. Harvey and E. Karlsson, Naunyn-Schmiedebergs Arch. Pharmakol., 312 (1980) 1–6.