Note

Separation of four cardiotoxins of Taiwan cobra (*Naja naja atra*) by reversed-phase high-performance liquid chromatography

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Snake cardiotoxin is a membrane-active polypeptide that causes irreversible depolarization of cell membrane[1]. There are four cardiotoxin (CTX) isomers in the venom of the Taiwan cobra (*Naja naja atra*), which are abbreviated to CTXI, CTXII, CTXIII and CTXIV, according to their sequential elution during purification on a CM-cellulose column. The amino acid sequences of all four isotoxins have been determined[2-6] and it is known that they are compact and basic proteins consisting of 60 amino acid residues with four disulphide linkages[7]. The major toxin, CTXIII, has been synthesized by solid-phase synthesis in our laboratory[8,9].

The separation of peptides and proteins by reversed-phase high-performance liquid chromatography (RP-HPLC) is a useful technique and many closely related proteins have been separated rapidly by this method[10]. It is necessary to have a rapid and accurate method for the measurement of synthetic polypeptides in the presence of the by-products commonly encountered during deprotecting and coupling procedures. RP-HPLC is not only rapid, compared with the use of conventional ion-exchange columns, but is also a powerful separation tool, giving resolution with a difference in only one of 50 amino acid residues[11].

Cardiotoxins are frequently used to investigate the destruction of artificial membranes[12,13]. Owing to the different activities of the four cardiotoxins, the cardiotoxin used as a research tool must be highly purified. The major toxin, CTXIII, when separated by conventional ion-exchange columns, was usually contaminated with CTXIV and was difficult to detect by disc electrophoresis[14,15].

In this paper, we compare the separations of the four CTX isomers achieved with four commercial reversed-phase columns. We have tried this method as a replacement for disc electrophoresis for checking the purity of CTX isomers purified by ion-exchange chromatography. It is also useful for monitoring the folding of reduced cardiotoxin under aerobic conditions.

EXPERIMENTAL

The cardiotoxins were purified from the crude venom of Taiwan cobra (*Naja naja atra*)[14], which was purchased from Cheng-Chin Co., Taiwan. The differences in the amino acid sequences of these toxins are listed in Table I. Acetonitrile (Alps Chem. Co., Taiwan) was of HPLC grade. Water for HPLC was prepared with a Milli-

TABLE I

DIFFERENCES IN AMINO ACID SEQUENCES IN THE FOUR CARDIOTOXINS

Cardiotoxin	Position													
	1	7	9	10	11	27	28	29	30	31	32	45	46	47
CTXI	Leu	Ile	Ile	Ala	Ser	Met	Ser	Asp	Leu	Thr	Ile	Ser	Asn	Leu
CTXII	Leu	Val	Leu	Phe	Tyr	Val	Ser	Asn	Leu	Thr	Val	Asn	Ser	Ala
CTXIII	Leu	Val	Leu	Phe	Tyr	Val	Ala	Thr	Pro	Lys	Val	Ser	Ser	Leu
CTXIV	Arg	Val	Leu	Phe	Tyr	Val	Ser	Asn	Leu	Thr	Val	Asn	Ser	Ala

Q water purification system (Millipore, Bedford, MA, U.S.A.). Sodium sulphate and sodium phosphate were purchased from Wako, Osaka, Japan.

The HPLC system consisted of two Waters Model 6000 pumps, a Waters UK-6 valve-loop injector, a Waters Model 450 variable-wavelength UV detector, a Waters refractive index detector, a Model 660 solvent programmer and an Omniscribe two-channel chart recorder. The Radial Compression Module, Radial-PAK C_{18} cartridges, C_8 cartridges, μBondapak-phenyl column and μBondapak-CN column were purchased from Waters Assoc., Milford, MA, U.S.A.

The experiments were performed at room temperature with a flow-rate of 1.5 ml/min. The detector was operated at 230 nm. The mobile phase, a mixture of acetonitrile and phosphate buffer containing sodium sulphate, was filtrated through a 0.45-μm membrane filter and degassed before use. Samples of about 30–50 μg were injected using a Microliter 802 syringe (Hamilton, Reno, NV, U.S.A.).

RESULTS AND DISCUSSION

The separation of a mixture of four isotoxins with a μBondapak-CN column is shown in Fig. 1. The order of elution of the four cardiotoxins was CTXIV, CTXII > CTXI > CTXIII. The two proteins, CTXIV and CTXII, could not be separated with this column, even though various conditions were investigated. CTXIV and CTXII differ only in one amino acid residue (position 1 = Arg in CTXIV and Leu in CTXII, as shown in Table I). Owing to the weak hydrophobicity of the μBondapak-CN column, CTXIV and CTXII may not be resolved. Using conventional ion-exchange CM-cellulose chromatography and disc electrophoresis, the two proteins were easily separated[1,4].

The four cardiotoxins were successfully separated on the μBondapak-phenyl column, as shown in Fig. 2. The phenyl group has a higher hydrophobicity than the CN group. The content of acetonitrile in mobile phase, therefore, was increased to
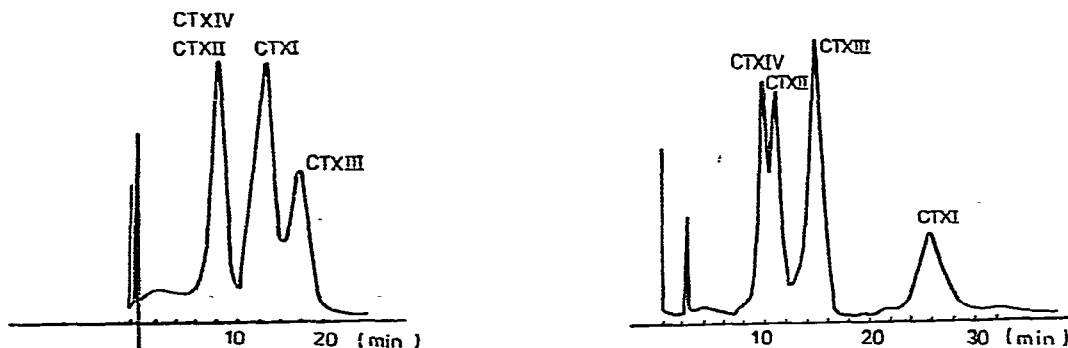

Fig. 1. Separation of the four cardiotoxins on a μBondapak-CN column. Eluent, mixture of 77% of 0.05 M phosphate buffer (pH 2.5) containing 0.05 M sodium sulphate and 23% of acetonitrile; flow-rate, 1.5 ml/min; detector, UV (230 nm).

Fig. 2. Separation of the four cardiotoxins on a μBondapak-phenyl column. Eluent, mixture of 71% of 0.05 M phosphate buffer (pH 2.5) containing 0.05 M sodium sulphate and 29% of acetonitrile; flow-rate, 1.0 ml/min; detector, UV (230 nm).
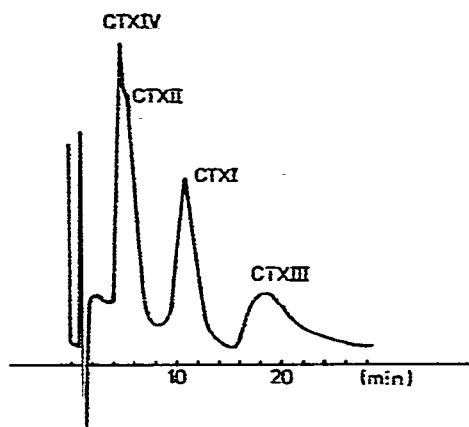
Fig. 3. Separation of the four cardiotoxins on a Radial Compression Module C$_8$ cartridge. Eluent, mixture of 70% of 0.01 M phosphate buffer (pH 2.5) containing 0.1 M sodium sulphate and 30% of acetonitrile; flow-rate, 1.5 ml/min; detector, UV (230 nm).
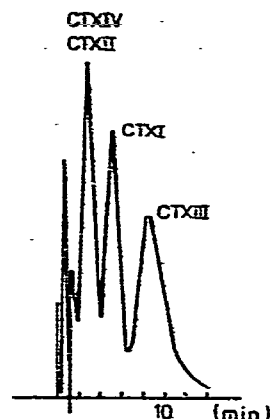
Fig. 4. Separation of the four cardiotoxins on a Radial Compression Module C$_{18}$ cartridge. Eluent, mixture of 67.5% of 0.01 M phosphate buffer (pH 2.5) containing 0.1 M sodium sulphate and 32.5% of acetonitrile; flow-rate, 1.5 ml/min; detector, UV (230 nm).

29% on the μBondapak-phenyl column in order to obtain a suitable retention time. It is surprising that the order of elution of the four cardiotoxins was CTXIV > CTXII > CTXIII > CTXI. The relative retention values of CTXI and CTXIII do not correspond to the relative hyophophobicities calculated from their amino acid residues (Table I). In general, the technique allows the separation of compounds on the basis of their hydrophobic interaction with the stationary phase support[16]. This simple rule seems to fit only small peptides (up to 20 amino acid residues), the hydro-
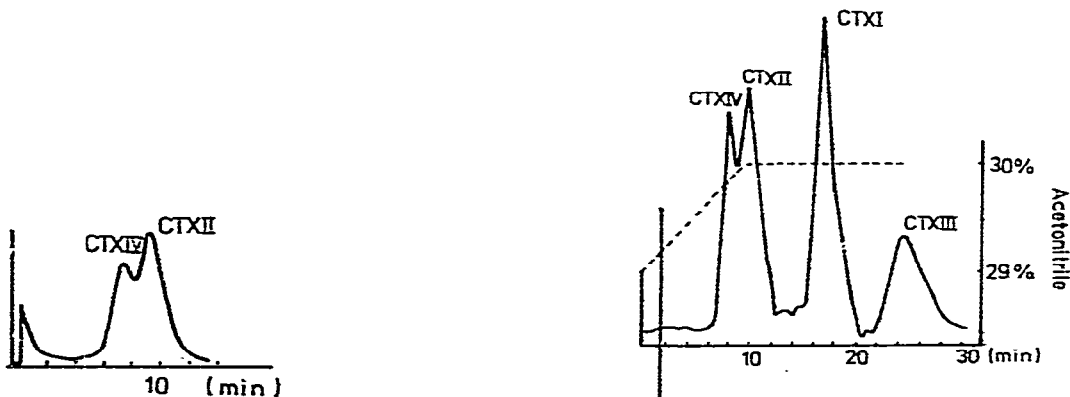

Fig. 5. Separation of CTXIV and CTXII on a Radial Compression Module C$_{18}$ cartridge. Eluent, mixture of 70% of 0.01 M phosphate buffer (pH 2.5) containing 0.1 M sodium sulphate and 30% of acetonitrile; flow-rate, 1.5 ml/min; detector, UV (230 nm).

Fig. 6. Separation of the four cardiotoxins on a Radial Compression Module C$_8$ cartridge. Eluent, mixture of 0.05 M phosphate buffer (pH 2.5) containing 0.1 M sodium sulphate and acetonitrile; a gradient from 29 to 30% of acetonitrile over 10 min was used; flow-rate, 1.5 ml/min; detector, UV (230 nm).

phobicity of which is determined by the sum of the contributions from each of their amino acid residues[17].

The orders of elution of the four cardiotoxins on the C_8 and C_{18} columns were the same (Fig. 3 and 4). CTXII and CTXIV cannot be separated on the C_{18} column if the acetonitrile concentration in mobile phase was higher than 32.5%. When the acetonitrile concentration reduced to 30%, CTXII and CTXIV were separated on the C_{18} column (Fig. 5), but the peaks were broad and still overlapped considerably. Using the C_8 column, CTXII and CTXIV were not separated if the mobile phase eluted under isocratic conditions (Fig. 3), unless a gradient from 29% to 30% acetonitrile in the mobile phase over 10 min was used (Fig. 6). The C_8 and C_{18} columns have such high hydrophobicities that CTXII and CTXIV could not be separated clearly.

From the results described above, it can be concluded that the μBondapakphenyl column is the best for the separation of the four cardiotoxins. The order of hydrophobicity of the four commercial columns is C_{18} > C_8 > μBondapak-phenyl > μBondapak-CN. Because of the moderate hydrophobicity of the μBondapakphenyl column, it is suitable for the separation of proteins that have high hydrophobicities, such as cardiotoxins. The elution conditions for the separation of cardiotoxins containing with very high percentages of acetonitrile, compared with the conditions required for cobra neorotoxin (only 15% of acetonitrile under the same conditions with the μBondapak-phenyl column)[18], also demonstrates that the hydrophobic groups in cardiotoxins were exposed to the surface of molecules, which are the character of membrane-active toxins[19].

Because of the similar net charges of XTCIII and CTXIV, their separation with an ion-exchange column is very difficult and tedious and the purity as checked by disc electrophoresis is uncertain. This problem was solved easily by using the RP-HPLC method.

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