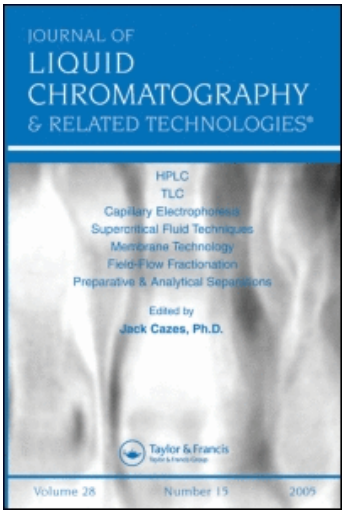

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SEPARATION OF TWO ISOFORMS (Ser^7/Thr^7) OF NATURAL SARAFOTOXIN-a BY CAPILLARY ELECTROPHORESIS: MASS SPECTROMETRY AND SYNTHESIS

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ABSTRACT

Each isoform was identified within the mixture in the natural sarafotoxin-a: Ser^7 and Thr^7-SRTX-a has been identified by chemical sequencing and recently by the sequencing of c-DNAs encoding for the sarafotoxins family. Mass spectrometry (MS) of the natural SRTX-a displayed the presence of two isoforms as noted by chemical and c-DNA sequencing. In order to evaluate the role of the 7 th residue in the bioactivity of SRTX-a , we carried out the separation of Ser^7 and Thr^7-SRTX-a from the natural SRTX-a isolated from the venom successicely by gel filtration, ion-exchange and reversed-phase HPLC. The capillary electrophoresis in micellar conditions (MECC) enabled us the separation of the Ser^7/ Thr^7-SRTX-a in an analytical range of peptide only. Due to the low range of the CE process in the quantitative recovery of two isoforms , each isoform was then synthesized

by solid phase for the bioassays and further spectroscopic studies. The MW and migration time of each synthetic isoform were found to fit with the corresponding natural product. Overall, CE (off-line in this study) with MS are the method of choice for the identification of natural products such as the SRTX-a isoforms because of its speed , nanogram scale sensitivity.

INTRODUCTION

The sarafotoxins(SRTX a,b,c,d) are a family of isopeptides (21 amino acids) , which were purified from the venom of the Israeli burrowing asp *Atractaspis engaddensis* (1) . These toxins have powerful cardiotoxic effects and induce the contraction of various smooth muscles.(2). A related family of cardiovascular and vasoconstrictor peptides, endothelins ET-1 , ET-2 , ET-3 , found in mammals and in other vertebrates (3) show a high degree of sequence homology with the sarafotoxin family.

The purification of SRTX-a from the venom successively by gel filtration, ion-exchange and reversed-phase HPLC and its sequencing by Edman degradation, enabled us to demonstrate the mixture of two isopeptides in the same HPLC peak: SRTX-a and SRTX-a1 which differ by only one residue (Thr/Ser) at the 7th position (4) (Figure 1). Furthermore, recent c-DNA cloning of the SRTXs family in our laboratory shows the existence of the two SRTX-a isopeptides namely SRTX-a1 or (Ser^7)-SRTX-a and SRTX-a or (Thr^7)-SRTX-a and also two SRTX-b isopeptides, namely SRTX-b1 or (Ser^7)-SRTX-b and SRTX-b or (Thr^7)-SRTX-b (5) . The different biological potencies of sarafotoxins have been attributed to the sequence heterogeneity in the N-ter region (4th to 7th residue) of the peptide (6), and specifically for the two most potent SRTXs (a and b) the difference between Ser^7 and Thr^7 raised a question on the role of the 7th residu on the biological potency of each isoform separately . Therefore, an attempt to isolate each natural isoform (SRTX-a / SRTX- a1) was carried out and the toxicity and bioactivity each isoform should be then tested to adress this question.

$Cys^{1}.Ser.Cys.Lys.Asp^{5}.Met.^{Ser}_{Thr}.Asp.Lys.Glu^{10}.Cys.Leu.Asn.Phe.Cys^{15}His.Gln.Asp.Val.Ile.Trp^{21}$

FIGURE 1

Amino-acid sequences of SRTX-a /(Thr^{7}) and a1 /(Ser^{7}) (disulfide bonds between Cys^{1} and Cys^{15}/ Cys^{3} and Cys^{11}) .

The analysis of the natural SRTX-a by mass spectrometry (MS) showed the presence of two isopeptides , as noted previously by chemical sequencing (4) . Capillary Electrophoresis (CE) only enabled us the separation of the two isopeptides. Due to the difficulty in scaling-up the separation of the two isoforms from the natural SRTX-a by CE , we decided to synthesize by solid phase the two isopeptides for the bioassays and further physico-chemical studies. In this paper, we wish to demonstrate 1/ the high resolving power and speed of the CE in the separation of two SRTX-isoforms and 2/ the usefulness of mass spectrometry and capillary electrphoresis in an off-line mode for the identification and characterization of the two SRTXs-a.

EXPERIMENTAL.

Purification of the mixture of two SRTX-a isoforms from the crude venom

The mixture of two SRTX-a isoforms was isolated and purified by column chromatography from the venom of *Atractaspis engaddensis* by gel filtration (Sephadex G-50) and ion-exchange (DEAE-cellulose) (4) . The fraction S6a of the DEAE-cellulose step was then purified by reversed phase chromatography on a Lichrosorb RP-18 column (250x4 mm, Merck, Damstadt, FRG) . The column was developed at 1 ml/min with a linear gradient of 0-80% acetonotrile in 0.1% trifluoroacetic acid using a Gilson model 303 HPLC system.

(^{252}Cf)-PDMS

Plasma desorption mass spectra were obtained with a DEPIL-(^{252}Cf)-time of flight mass spectrometer (7) at the Institut de Physique Nucléaire

(Orsay, France). The sample preparation method used is devised by Sundquist et al. (8). The peptide solution (10μL) was applied to a nitrocellulose target obtained by electrospraying a nitrocellulose solution onto an aluminized mylar foil. The nitrocellulose target was allowed to absorb the peptide solution (5 min.) and then spin dried. The concentration of peptide used for the PDMS analysis was estimated by UV spectra: 1μg/mL to 10 μg/mL. Therefore, the quantity of the analyzed peptide was 10 ng to 100 ng for the deposit of 10 μL. The target was then submitted to fission fragment in the mass spectrometer without being rinsed with water. The variable time of flight distance was set to 40 cm and the accelerating voltage to 15 kV. All spectra were collected for 15 min. in the positive mode, although the molecular peaks were clearly identifiable within a few seconds. Mass values are calculated from the centroids of time of flight peaks (7).

Capillary Electrophoresis (CE)

The separation of the two natural isopeptides and the elution of synthetic peptides were carried out on the ABI 270 A-HT apparatus (ABI, USA) with a 500 mm x 50 μm i.d. fused-silica capillary. Samples were introduced from the cathodic end of the capillary by 1 s. vacuum injection. Peptide solutions were prepared to be about 1 μg/ mL and the volume of one peptide solution injected corresponded approximately to a quantity of 10 ng of peptide. UV detection was at 200 nm. Before each run the capillary was washed with 1.0 M NaOH for 2 min followed by wash with buffer for 5 min.

Micellar electrokinetic capillary chromatography (MECC) conditions were based on the use of a surfactant concentration of 50 mM SDS in running buffer of 50 mM sodium tetraborate pH 8 (9). The experimental conditions are described in the legends of the figure.

Solid Phase Peptide Synthesis

SPPS was carried out on the automatic synthesizer (ABI 430 A) using the Boc-chemistry (10) and the two disulfide bonds were formed seqentially by using a specific chemistry : ferricyanide oxidation of Cys^3 and Cys^{11}

(11) and iodine oxidation of Cys(Acm)^{1} and Cys(Acm)^{15} (12). Boc-(L)-Trp(For)-Pam-Resin (0.66 mM/g) was used as the starting resin. The linear peptide Cys^{3,11} and Cys(Acm)^{11,15} was cleaved from the resin and purified by reversed phase HPLC. The mono-disulfide peptide was obtained after the ferricyanide oxidation and HPLC purification steps. The bi-disulfide-peptide or SRTX-a was obtained after the iodine oxidation and HPLC purification steps. The synthetic peptides were characterized by amino acid analysis , PDMS and CE.

RESULTS AND DISCUSSION

The mass spectrometry measurements of the purified natural SRTX-a displayed one protonated molecule (MH^+) observed at m/z 2511.79 corresponding to the (Thr^{7})-SRTX-a (m/z calc 2512.90) and other protonated molecule (MH^+) observed at m/z 2497.62 corresponding to the (Ser^{7})-SRTX-a (m/z calc. 2498.60). The difference in mass (14 a.m.u.) fit in with the substitution of one H atom from the serine side-chain by one CH_3 group for the threonine side-chain (Figure 2-A). Two other peaks with a minor intensity at m/z 2466.52 and m/z 2452.37 (difference of 45 a.m.u. respectively from the two major peaks) can not be assigned as a product of fragmentation from the two previous isoforms. The identity of these two peaks was not studied, but we thought that these two MH^+ values can likely correspond to the isoforms of the respective natural (Thr^{7})-SRTX-a and natural (Ser^{7})-SRTX-a.

In the first step, we tried to separate the two natural isopeptides by C.E. for bioassay measurements. Classical conditions of separation of (Ser^{7})-SRTX-a and (Thr^{7})-SRTX-a by C.E. were not successful. Different conditions were extensively investigated in the optimization of the resolution of the two natural isoforms: acidic, alkaline buffers, buffer concentration and applied voltage. C.E. in micellar conditions (MECC) e.g. in the presence of the detergent SDS, enabled the separation of the two components in the natural SRTX-a : the (Thr^{7})-SRTX-a migration time is longer to that of (Ser^{7})-SRTX-a (Figure 2 B). Two minor peaks at 7.92 min and 7.74 min are observed as in

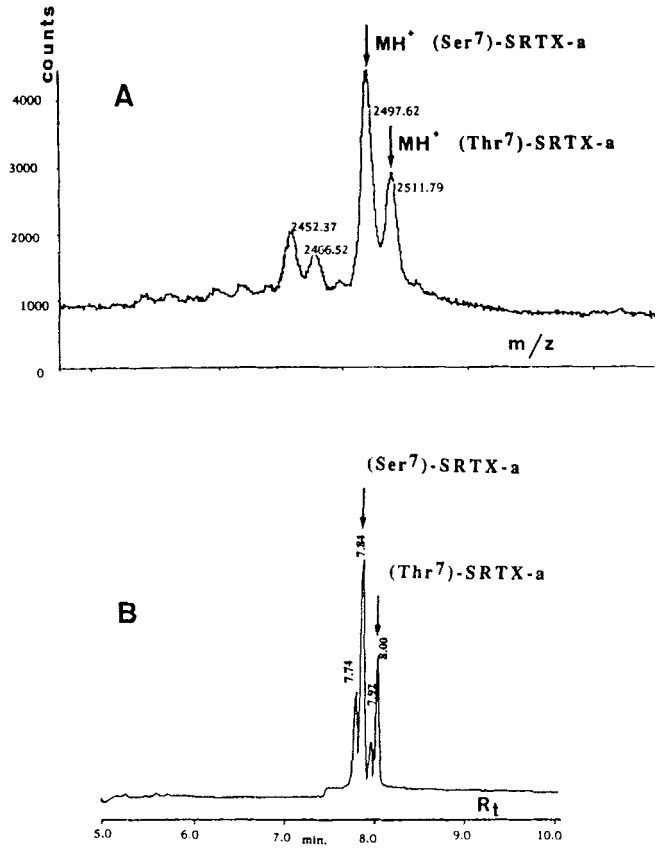

FIGURE 2

A- (^{252}-Cf)-PDMS of natural SRTX-a purified from the *Atractaspis engaddensis* venom. The synthetic (Ser^7)-SRTX-a and (Thr^7)-SRTX-a positions' on the respective mass spectrum are indicated by the arrows.

B- CE in MECC conditions of natural SRTX-a purified from the *Atractaspis engaddensis* venom. The synthetic (Ser^7)-SRTX-a and (Thr^7)-SRTX-a positions' on the respective capillary electrophoregram are indicated by the arrows. The experimental conditions are : capillar lenght L=50 cm ; 15 kV ; 38 μA ; borate 50 mM pH 8.0 ; SDS 50 mM . Detector at 200 nm , 30°C. Samples are dissolved in 4 mM sodium citrate buffer at pH= 2.5.

the PDMS spectrum and can be likely assigned as the assumed isoforms of (Thr^7)-SRTX-a and (Ser^7)-SRTX-a (Figure 2B). This separation can be considered as the result of a difference in the partitioning of (Ser^7)/(Thr^7)-SRTX-a into the micelle (9) . At this point, it seems to us that the secondary structure of SRTX-a must be taken into account for the partitioning of the two isoforms into the micelle. The 6th-7th residues in the SRTX-b lying between the 3th-6th β-turn region and the helicoidal core (8th-15th region) are believed to have a β–sheet preference according to the NMR study of the SRTX-b (13) , an analog of the SRTX-a , peptide which has the same sequence from the 1st to the 12th residue (4). The Ser^7 or Thr^7 side-chain in SRTX-a should be exposed to the solvent and the detergent , which in turn facilitates the separation of the two isoforms : the greater the hydrophobicity of the side-chain , the longer the migration time (R_t) . The recovery of purified sample by CE for further studies is usually not quantitative. Therefore, we synthesized the two isopeptides SRTX-a and a1. The details of the solid-phase peptide synthesis will be published elsewhere. Mass spectrometry and the CE migration time of each synthetic isopeptide were compared individually to the natural product (Figure 2 A and B). Each synthetic peptide's MH^+ and R_t values are exactly at the same values to those of the corresponding natural product. The synthetic peptide's behaviour in MECC is identical to that of the natural product.

These CE results in MECC conditions demonstrate the high resolving power of the method :1/ good resolution of the 21 residues-peptide which differes only by one methyl group (serine / threonine side-chain) 2/ need of low quantity of natural product (nanogram scale). Used in combination with mass spectrometry, off-line in our study, the CE method seems to be ideal for the purification, identification and characterization of natural products on a nanogram scale.

The toxicity of each peptide was then measured. Preliminary results on the toxicity of each SRTX (a and a1) show an identical LD_{50} for both peptide (10 ng/g b.w. in mice) . This results suggest that the mutation of the 7th residue (Thr-Ser) didn't affect the toxicity of the SRTX-a. The bioactivity of each isoform is under study.

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ABBREVIATIONS

PDMS : plasma desorption mass spectrometry ; C E : capillary electrophoresis; MECC : micellar electrokinetic capillary chromatography ; SPPS : solid phase peptide synthesis ; acm : acetamidomethyl ; For : formyl ; Pam : phenylacetamidomethyl ; a.m.u.: atomic mass unit.

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