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Detection of isoforms and isomers of rattlesnake myotoxins by capillary electrophoresis and matrix-assisted laser desorption time-of-flight mass spectrometry

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Abstract

Capillary electrophoresis (CE) was used to study myotoxins, members of a highly homologous family of small, basic, non-enzymatic proteins found in rattlesnake venoms. Several rattlesnake species were investigated and conditions were set to optimize the one-step separation of the myotoxins from the rest of the venom components. Myotoxin containing venoms, when subjected to CE analysis, yielded multiple myotoxin peaks. The myotoxin fractions were collected and further analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry. In some instances, multiple mass peaks were observed from the mass spectra, suggesting the existence of myotoxin isoforms. Furthermore, each venom that contained myotoxin yielded fractions with indistinguishable masses, indicating that myotoxin isomers were present. The isomers were readily detected in the electropherograms since they exist in a ca. 1:4 ratio, data already established for myotoxin *a* from the prairie rattlesnake. An in-column incubation method was used to address the re-equilibration of the separated isomers. © 1997 Elsevier Science B.V.

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1. Introduction

Myotoxins are a group of closely related, small, protein toxins found in venoms from several rattlesnake species [1]. These toxins cause extensive damage to skeletal muscle tissue upon envenomation [2]. Sequences for five members of this group have been published and the sequence similarities range from 83% to 98% [3]. In addition, presence of myotoxin isoforms was established for several of the myotoxins, the most evident being the existence of multiple myotoxin sequences from the venom of a single prairie rattlesnake [4]. Myotoxin *a* [5] is the most studied member of this group of toxins. Recent structural studies [6,7] revealed an existence of conformational heterogeneity in a sample of myotoxin *a*. Reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) separations of a purified myotoxin *a* gave rise to two resolved peaks in approximately 1:4 ratio. Each fraction, when collected, incubated and re-analyzed, yielded the original separation profile of the myotoxin *a* sample. The two forms of myotoxin have the same mass and it was suggested that they represent conformational isomers of myotoxin *a* that are result of a *cis–trans* isomerization around the Ile19–Pro20 peptide bond [6]. The sample heterogeneity of myotoxin *a* has been a major obstacle in our attempts to generate a well defined three-dimensional structure.

CE is a powerful tool for the study of proteins [8]. It is a rapid and sensitive analytical technique that is

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widely used for separation of molecules that differ slightly in their sequences or conformations. Separation of closely related isoforms by CE has been achieved for a variety of proteins [9–12]. CE was also successfully used to separate protein conformers that differ only in their disulfide bridge arrangement [13]. More recently, a series of L-Ala and D-Ala substituted polypeptide diastereomers were separated in untreated fused-silica capillaries [14]. *Cis–trans* isomerization of a peptidyl–proline bond has also been studied by CE. Separation of *cis–trans* isomers of dipeptides [15,16] and of some small polypeptides [16,17] has been successfully achieved.

In this study, CE, together with matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS), was used to address the question whether the conformational heterogeneity discovered in myotoxin *a* is also found in the rest of the proteins belonging to the myotoxin family. Instead of pure samples of myotoxins, venoms from four rattlesnake species for which myotoxin sequences have been published were investigated and a one-step CE separation of the myotoxins in the venoms was developed. Fractions corresponding to the myotoxins were collected and analyzed by MALDI-TOF-MS. Accurate mass determination was achieved using the whole venoms as MALDI samples. In addition, in-column interconversion of the separated fractions was investigated.

2. Experimental

2.1. Chemicals and samples

Lyophilized *Crotalus viridis viridis* crude venom was obtained from the Western Institute of Biomedical Research (Salt Lake City, UT, USA), *Crotalus durissus terrificus* venom from Miami Serpentarium Laboratories (Salt Lake City, UT, USA), *Crotalus adamenteus* and *Crotalus viridis helleri* venoms were gifts from the late James Glenn (Venom Research Lab., Veterans Administration Medical Center, Salt Lake City, UT, USA). All venoms were dissolved in triple distilled water and centrifuged at high speed for 15 s to remove any insoluble components. Sodium hydroxide was purchased from EM Science (Gibbstown, NJ, USA), acetic acid from JT Baker (Phillipsburg, NJ, USA), α-cyano-4-hydroxycinnamic acid and polyethyleneimine (average M_r of 750 000) from Aldrich (Milwaukee, WI, USA), trifluoroacetic acid from Pierce (Rockford, IL, USA), acetonitrile from Fisher Scientific (Fair Lawn, NJ, USA) and cytochrome *c* from Sigma (St. Louis, MO, USA).

2.2. Capillary electrophoresis

A P/ACE System 2050 (Beckman Instruments, Fullerton, CA, USA) was used for the CE experiments. A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 67 cm total length (60 cm effective length)×50 μm I.D.×360 μm O.D. was coated with 0.1% polyethyleneimine (PEI) according to the procedure described by Erim et al. [18]. A 3 min washing step with buffer was used between injections, occasionally preceded by water and air rinses (3 min each) in order to reestablish the original electroosmotic flow. The running buffer used in all separations was 0.1 *M* acetate (2.0 *M* acetic acid adjusted with 10.0 *M* sodium hydroxide to pH 4.0 and diluted to 0.1 *M* concentration). Samples were injected under pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) at the cathodic end of the capillary and a running voltage of 20 kV was applied (yielding a current between 9 and 12 μA). The temperature was thermostatted at 25°C and detection was at 214 nm. Fractions were collected by stopping the runs at specific times (determined by calculating the sample mobility at the UV window and extrapolating it to the end of the capillary). The desired peak(s) were collected under high-pressure mobilization into a vial containing 10 μl water. The collected fractions were directly subjected to mass analysis without any post-collection manipulation.

2.3. MALDI-TOF-MS

A LaserTec Research MALDI time-of-flight mass spectrometer (PerSeptive Biosystems, Cambridge, MA, USA) was used to analyze the fractions collected from CE. A saturated solution of α-cyano-4-hydroxycinnamic acid in 1.5% trifluoroacetic acid–acetonitrile (2:1, v/v) was used as a matrix. Sample solutions and the matrix (2 μl each) were directly mixed onto the probe tip. Accurate mass determi-

nations of the myotoxins collected from the CE were complicated by their sub-picomole concentrations and by signal suppression from an internal standard. To avoid these problems, the myotoxin masses were determined using samples of whole venoms premixed with cytochrome *c* (M_r=12 360.7) which served as a standard for internal calibration. Careful comparisons of the mass spectra of each whole venom and its corresponding CE collected fractions were done before assigning a mass to each fraction.

3. Results and discussion

The highly basic character of the myotoxins (p*I* values above 10.0, Ref. [7]) presents a major difficulty in their analysis by CE. Unsuccessful attempts to separate different forms of myotoxins were made using both untreated fused-silica capillaries and deactivated silica capillaries (unpublished results). In order to avoid the interactions between the capillary wall and the myotoxins, a polyethyleneimine (PEI) coated capillary for separation of basic proteins was used [18]. A 0.1% PEI concentration yielded the highest EOF and was utilized for all the experiments. A 50 μm capillary I.D. gave the most satisfactory and reproducible results. Capillaries with larger diameters (75 and 100 μm I.D.) were tested in attempt to increase the sample load, but were very unstable, presumably due to the increased Joule heating (results not shown).

CE separations of whole venoms from two rattlesnake species, *Crotalus viridis viridis* and *Crotalus durissus terrificus* are illustrated in Fig. 1. The myotoxins, being the most basic components in the venom, migrated most rapidly towards the cathode, which in turn resulted in slowest apparent electrophoretic mobility. In each case, the myotoxins were well separated from the rest of the venom components. The myotoxin fractions were collected and subjected to MALDI-TOF-MS experiments which are shown as small insets in Fig. 1. The myotoxin in the *C. v. viridis* venom shows two peaks (labeled 1 and 2) in the CE separation and only one in the mass spectrum (Fig. 1a). The mass of the two peaks corresponds well with the theoretical mass for myotoxin *a* (Table 1) which is the major myotoxin component in the *C. v. viridis* venom. The existence

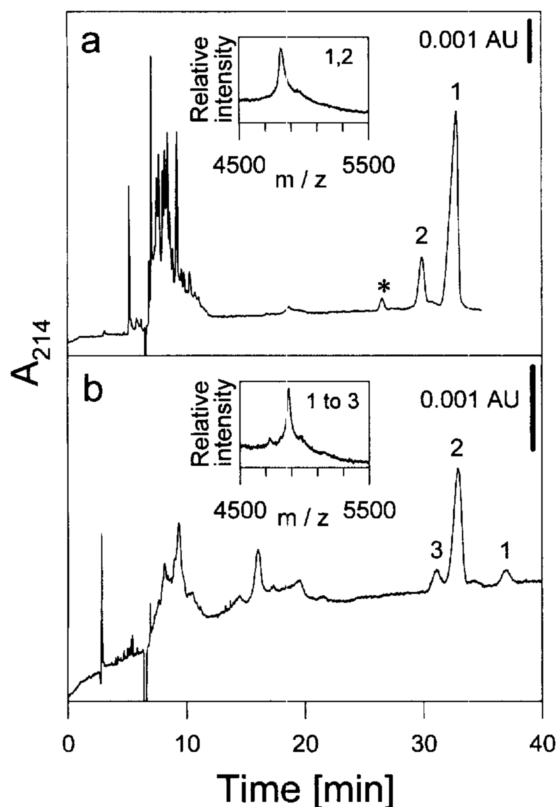

Fig. 1. CE separation of *Crotalus viridis viridis* (a) and *Crotalus durissus terrificus* venom (b). Conditions: 67 cm×50 μm I.D., 0.1% PEI coated capillary; 0.1 *M* acetate buffer pH 4.0; voltage, 20 kV; temperature, 25°C. Sample concentration and injection, 1 mg/ml, 5 s (a) and 2 mg/ml, 20 s pressure injection (b). Insets: MALDI-TOF-MS analysis of fractions 1 and 2 (a) and 1, 2 and 3 (b) collected from the CE separations.

of the two peaks in a ca. 1:4 ratio is consistent with the separation profile of a purified sample of myotoxin *a* [6,7] and represents sound evidence for the existence of the two isomers in the whole venom. The mass of the small fraction labeled with an asterisk was not determined because of the small amount in the collected fractions.

Three major myotoxin peaks were seen in the CE separation of the *C. d. terrificus* venom (Fig. 1b). Peaks 2 and 3 correspond to crotamine (Table 1), a well characterized myotoxin with amino acid sequence that differs from the myotoxin *a* sequence in only three positions [19]. The area ratio of these two peaks is similar to the one seen for myotoxin *a*.

Table 1
Identification and molecular mass determination by MALDI-TOF-MS of the peaks separated by CE

Venoms	CE peaks	M_r (exp.)	Myotoxin (Ref.)	M_r (theor.)[a]	Error (%)
C. v. viridis	1, 2	4821.7	Myotoxin *a* ([5])	4821.7	0
C. d. terrificus	1	4736.9	–	–	–
	2, 3	4880.1	Crotamine ([19])	4885.7	0.1
C. v. helleri	1, 2	4876.4[b]	–	–	–
	3, 4	4967.7[d]	Peptide c ([22])	4982.9	0.3
	5, 6	5159.9[c]	–	–	–
	7	4222.9	–	–	–

[a] Obtained from the SWISS-PROT annotated protein sequence database (http://expassy.hcuge.ch).
[b] Similar to crotamine (0.2% error).
[c] Similar to *C. v. viridis* myotoxin "*viridis*-3" (M_r=5168.1, Ref. [4]) (0.2% error).
[d] Possible amino acid subsitution would be the reason for the observed mass difference.

Therefore, it is very possible that these two fractions represent conformational isomers of the same kind as the ones seen in myotoxin *a*. The identity of fraction 1 could not be established directly because of its small abundance in the sample (the mass spectra of fraction 1 did not yield any peaks). An attempt to increase the sample load was made but resulted in a loss of peak resolution. Nevertheless, both the mass spectra of the three peaks together (Fig. 1b inset) and of the whole venom (not shown) revealed a peak with a mass smaller than crotamine. This mass value was assigned to peak 1 (Table 1). Whether it represents a myotoxin was not established in this study. The mass value of this peak is smaller than the lowest myotoxin mass (myotoxin *a*) published so far.

The CE separation of *Crotalus adamenteus* venom did not show any myotoxin peaks, neither did the mass spectrum from the whole venom (results not shown). The sequence of a myotoxin from venom of this species (CAM toxin) has already been published [3]. The absence of myotoxins in this venom can be explained by the geographical origin of the venom tested, since similar results were observed in a previous study [20].

A CE analysis of *Crotalus viridis helleri* and mass spectra of selected myotoxin fractions are shown in Fig. 2. Multiple peaks were observed at migration times that correspond to myotoxins. Mass analyses of the fractions helped determine the identity of each of the seven peaks (see figure caption and Table 1). So far, only one sequence has been published for a

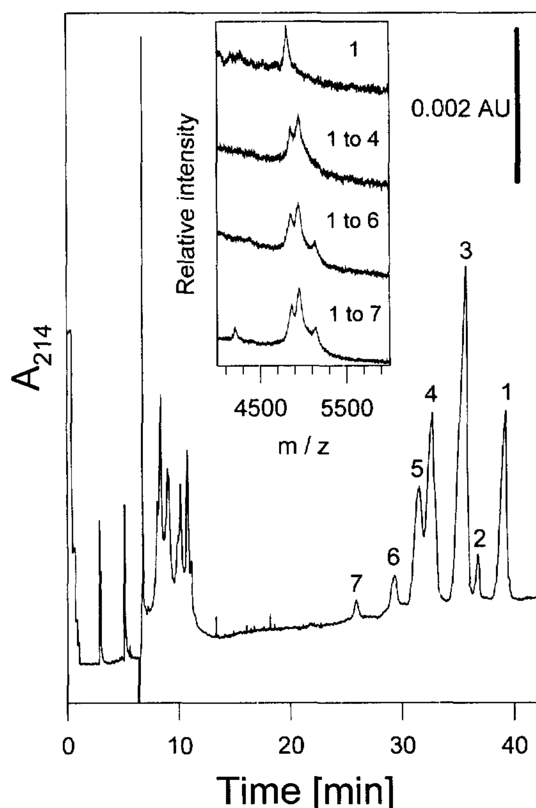

Fig. 2. CE separation of *Crotalus viridis helleri* venom. Conditions as in Fig. 1 except 5 mg/ml sample injected for 5 s. Several CE runs were performed in which different fractions were collected. Inset: MALDI-TOF-MS of various myotoxin fractions collected from the CE separations of the *C. v. helleri*.

myotoxin from *C. v. helleri* venom [21], but our results indicate the existence of three different isoforms (fraction 7 was not considered to be myotoxin). Each isoform shows two peaks in the CE separation in a ratio close to values observed for crotamine and myotoxin *a*. This is an indication of the existence of conformational isomers for each of the different myotoxin sequences present in the venom.

The existence of two interconverting myotoxin *a* isomers has already been established by isolation of each fraction and analysis of each individual fraction using both CE and RP-HPLC [6]. Recently, a new method was developed for studying the kinetic parameters of the *cis–trans* isomerization of five proline-containing dipeptides [22]. This method, named "in-column incubation", was used in this study to investigate the interconversion of the myotoxins present in whole venoms. Fig. 3 shows the re-equilibration of myotoxin *a* from a sample of *C. v. viridis* venom. Some interconversion was observed after 1 h incubation (Fig. 3b) and it was complete after 12 h (Fig. 3c), as seen from the rise of the middle peak (labeled with an "▼"). An estimate of the kinetic parameters was not possible because the "newly" formed fractions were not resolved and appeared as a single peak in the electropherogram. The short capillary length available for separation

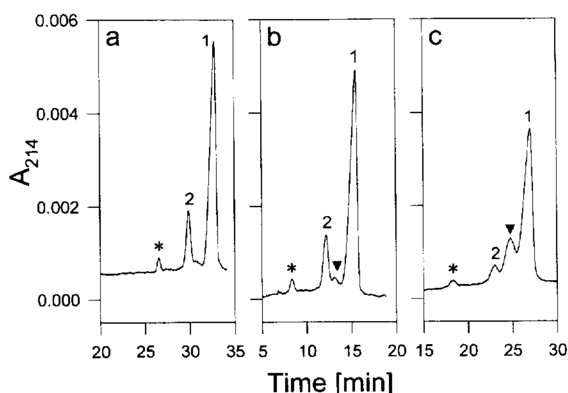

Fig. 3. CE in-column incubation and separation of myotoxin *a* from *Crotalus viridis viridis* venom sample. Conditions and peak labeling: as in Fig. 1a. Normal (control) separation (a); separation after 1 h in-column incubation of a sample separated by a run stopped at 20 min (b); separation of a sample incubated for 12 h from a run stopped at 15 min. The new peak that resulted from the interconversion of fractions 1 and 2 is labeled with an "▼".

after the incubation and the broadening of the peaks contributed to the merging of the converted fractions. The same method was tried with crotamine from the *C. d. terrificus* venom (results not shown). Crotamine showed a similar pattern of interconversion but none of the peaks were well resolved after the incubation.

4. Conclusions

CE was successfully used to separate both isoforms and conformational isomers of several myotoxins from venoms of different rattlesnake species. The one-step CE separation of the myotoxins represents a new, fast method of establishing their presence in the venoms. It appears that the conformational heterogeneity originally observed in myotoxin *a* is a common occurrence among myotoxins. This comes as no surprise since all the myotoxins exhibit high sequence similarity. Furthermore, the region of the sequence suggested to be responsible for the isomerization (Ile19–Pro20) [6] is conserved in all published myotoxin sequences [1]. The in-column incubation method confirmed the existence of two interconvertible forms of myotoxin *a* and crotamine in solution.

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