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Proteomics of the venom from the Amazonian scorpion *Tityus cambridgei* and the role of prolines on mass spectrometry analysis of toxins

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Abstract

Scorpion venom are complex mixtures of peptides, known to cause impairment of ion-channel function in biological membranes. This report describes the separation of approximately 60 different components by high performance liquid chromatography and the characterization by Edman degradation and mass spectrometry of 26 peptides from the soluble venom of the Amazonian scorpion *Tityus cambridgei*. One of these peptides, named Tc48a, was fully characterized. It contains 65 amino acid residues, the C-terminal residue is amidated and it affects Na^+-channels with a K_d of about 82 nM. Furthermore, this report shows the thermo-instability of scorpion toxins subjected to electron spray ionization-mass spectrometry (ESI-MS). When a proline residue is located near the N-terminal region of the toxin, not stabilized by disulfide bridges, artificial components are generated by the mass spectrometer conditions, due to the cleavage of the peptide bond at the proline positions. This phenomenon was confirmed by using four model proteins (variable regions of immunoglobulins) studied by ESI-MS and matrix assisted laser desorption ionization–time of flight (MALDI–TOF)/MS.

Keywords: Proteomics; *Tityus cambridgei*; Toxins; Proline

1. Introduction

The venom of scorpions is a very complex mixture of peptides, most of them known to be toxic to various organisms, but also contain enzymes, nucleotides, lipids, biogenic amines, and other unidentified components. The toxic fractions usually affect ion-channels of excitable and non-excitable cells. They are classified according to structural and physiological effects into several families and subfamilies of distinct peptides [1,2]. The classical biochemical approach to study scorpion toxins is based on their purification to homogeneity by sequential steps of chromatographic techniques, followed by primary structure determination using automatic Edman degradation [1]. The expression levels of the complex pool of toxins produced by the venomous glands of scorpion are dependent on many different factors such as: genetic variations, geographic areas, elapsing predatory time, gender, and other environmental conditions [3]. Comparison of chromatographic profiles obtained from total venom of the same species of scorpion, sometimes reveals variations on the quality of the chromatogram. Some components might disappear or may be hidden in the complexity of the sample, making difficult to identify them, thus affecting the entire and precise proteomics of the venom.

In the 1980s, the introduction of the techniques of electron spray ionization-mass spectrometry (ESI-MS) and matrix assisted laser desorption ionization–time of flight (MALDI–TOF), which made possible to produce ionized derivatives of macromolecules, changed completely the way in which biochemical analysis of toxins can now be conducted. Impure samples from liquid chromatography (on-line or off-line) can be isolated and analyzed using adequate alternate current voltage and radio-frequency in mass spectrometers. A significant number of powerful technologies coupled to mass spectrometry analysis have been developed recently. Multidimensional Protein Identification

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Technology (MudPIT) is a high-throughput method for protein identification from very complex mixtures using a biphasic column (ion-exchange and C_{18} reversed phase column), which enables to identify hundreds of proteins as well as post-translational modifications [4,5]. The isotope-coded affinity tags (ICAT) is a method based on a class of new chemical reagents and MS which can identify complex mixtures of proteins and quantitatively measure the differences in protein expression from different tissues or organisms [6]. Moreover, the identification of *N*-glycosylation sites using ^{18}O-label and peptide-*N*-glycosidase F deglycosylation [7], and the miniaturized immobilized metal (Fe^{3+} and Ga^{3+}) affinity chromatography (IMAC) columns for the enrichment of phosphopeptides and posterior MS detection [8] among others, have increased the range of MS applications in medical and biological sciences.

Thanks to the emerging technologies and to the tremendous increase in available computing power, the proteomic analysis of scorpion venoms can be performed easily and seems to have just started. Still to go, there are *circa* 1500 different species of scorpions, containing at least 100,000 different biomolecules with potential pharmacological applications waiting to be separated and studied [1]. At present, no more than 300 distinct peptides have been isolated and structurally characterized from about 30 different species of scorpions. The genus *Tityus* is now being studied by the use of mass spectrometry measurements [9], but from many species there is nothing published in the literature.

The chemical composition of *Tityus cambridgei* venom is poorly known, despite the fact that it is the most dangerous Amazonian scorpion and many serious envenomation cases of humans have been reported (Figueiredo, Carvalho, in: http://www.ufmt.br/niefa/perfilacidentesescorpi.html; and Campos Filho, in: http://www2.uol.com.br/JC/_1999/1403.htm).

In this work we describe the mass fingerprint of the venom from the Brazilian scorpion *T. cambridgei*, partial amino acid sequence of 26 toxins, and the full sequence of the Tc48a toxin, including its electrophysiological effect on Na^+-channels. Mass spectrometry results from ESI-MS and MALDI–TOF/MS provided interesting results on the thermo-stability of the toxins containing proline residues at the N-terminal region, that are not linked to other segments of the peptide by adjacent cysteine residues involved in disulfide bridge formation. In order to add proof to our interpretation, a series of experiments were performed by using model proteins (mutants of variable regions of immunoglobulins) which are reported here for the first time.

2. Experimental

2.1. Source of venom and chemicals

The venom of *T. cambridgei* was obtained by electrical stimulation, from scorpions collected in the State of Amazonas, Brazil (see Acknowledgements). All solvents and chemicals used were analytical grade and double-distilled water was used throughout.

2.2. Isolation procedures

The venom was solubilized in water and spun at $10{,}000 \times g$ for 5 min. One hundred microliters containing 1.0 mg of the soluble venom was directly submitted to an analytical C_{18} reversed phase column (250 mm × 10 mm) obtained from Vydac (Hisperia, CA, USA). Components were purified using a linear gradient from solution A (0.12% trifluoroacetic acid (TFA) in water) to 60% solution B (0.10% TFA in acetonitrile), run for 60 min. The detection was monitored by absorbance at 230 nm with 0.5 U sensitivity and eluted at 1 ml/min flow-rate. Fractions were collected manually and dried using a Savant Speed-Vac drier.

2.3. Amino acid sequence determination

Amino acid sequence determination of pure toxins and fragments of the alkylated toxins after digestion with several enzymes was performed by automatic Edman degradation in a Beckman LF 3000 Protein Sequencer (Palo Alto, CA, USA) using the chemicals and procedures previously described [10]. The enzymes used for this work were endopeptidase lysine-C (Lys-C) and endopeptidase V8 from *Staphyloccocus aureus* (Glu-C) from Boehringer (Mannheim, Germany).

2.4. Mass spectrometry analysis

2.4.1. ESI-MS

The components from LC were reconstituted to a final concentration of 500 pmol/5 μl of 50% acetonitrile with 1% acetic acid and directly applied into a Finnigan LCQ^{DUO} ion trap mass spectrometer (San Jose, CA) using a Surveyor MS syringe pump delivery system. The eluate at 10 μl/min was splitted in order to allow only 5% of the sample to enter the nanospray source (0.5 μl/min). The spray voltage was set from 1.0 to 2.0 kV and the capillary temperature was set from 100 to 200 °C depending on the chosen experimental conditions. For MS experiments, the fragmentation source was operated with 25–35 V of collision energy, 35–45% (arbitrary units) of normalized collision energy and the scan with wide band activated. All spectra were obtained in the positive-ion mode. The data acquisition and the deconvolution of data were performed on Xcalibur Windows NT PC data system. The MS/MS spectra from peptides enzymatically generated were analyzed manually and by Fuzzy ions, an utility of Sequest software obtained through Finnigan (San Jose, CA).

2.4.2. MALDI–TOF/MS

MALDI–TOF/MS analysis was performed on an Autoflex mass spectrometer (Bruker Daltonics, Billerica, MA,

USA) in the positive linear and reflector mode. The samples in triplicate were spotted onto the target at variable concentrations followed by addition of three different matrices for each protein: α-cyano-4-hydroxycinnamic acid (CHCA), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 2,5-dihydroxybenzoic acid (DHB). Nitrogen laser at 337 nm was used to desorb the samples with intensity from 50 to 100% (arbitrary units).

2.5. Electrophysiology

2.5.1. Cell culture

Cells of the F-11 clone (mouse neuroblastome N18TG-2 × rat DRG) [11] and cells of the TE671 clone [12] were routinely cultured in Dulbecco's modified Eagle medium (DMEM), containing 4.5 g/l of glucose and 10% fetal calf serum. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.5.2. Solutions

The standard extracellular solution contained (mM): NaCl 130, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, HEPES–NaOH buffer 10, D-glucose 5, at pH 7.40. The standard pipette solution at $[Ca^{2+}]_i = 10^{-9}$ M (*p*Ca 9) contained (mM): K^+-aspartate 130, NaCl 10, $MgCl_2$ 2, EGTA–KOH 10, HEPES–KOH buffer 10, ATP (Mg^{2+} salt) 1, pH = 7.30. The pipette solution contained (mM) K^+-aspartate 110, KCl 23, $CaCl_2$ 0.4 (*p*Ca = 7), $MgCl_2$ 3, HEPES–KOH buffer 5, GTP (Na^+ salt) 0.4, ATP (Na^+ salt) 5, creatine phosphate 5, pH = 7.3. Toxins were directly dissolved into the extracellular solutions from a stock solution.

2.5.2.1. Patch-clamp recordings. The currents were recorded at room temperature as previously described [13]. Pipette resistance (1–2 MΩ), cell capacitance and series resistance errors were carefully compensated (85–95%) before each voltage clamp protocol run. The extracellular solutions were delivered through a 9-hole (0.6 mm) remote-controlled linear positioner placed near the cell under study, which has an average response time of 5–8 s. Currents were recorded by means of a MC700A patch-clamp amplifier (Axon Instruments, Foster City CA, USA). Protocol pulses were delivered every 12 s and holding potential was set at −60 mV. All currents were corrected by subtracting recordings done in 200 nM TTX. During data acquisition and analysis, pClamp 8.2 (Axon Instruments, USA) and Origin 4.1 (Microcal Inc., USA) software were routinely used.

2.6. Protein 6aJL2 mutants

The protein 6aJL2 is a V_L domain carrying lambda 6-germ line sequence. Its gene was synthesized by recursive polymerase chain reaction (PCR) using eight overlapping oligonucleotides that include the restriction sites of SfiI and NotI enzymes (del Pozo et al., in preparation). The PCR product was cloned in the plasmidic vector pSyn1 and expressed in HB2151 strain of *E. coli* cells [14]. The expressed protein was recovered from the periplasmic fluid using the method described before [14], and it was purified by means of a three steps strategy: (1) precipitation of protein with 74% ammonium sulphate solution, (2) chromatographic purification in a Superdex 200 High Load column, and (3) HPLC separation with a C4 reversed phase column, from Vydac (Hisperia, CA), using a gradient from 0 to 40% acetonitrile in 0.1% TFA. Bona fide lambda 6-germ line sequence of 6aJL2 was determined at DNA and protein levels (del Pozo et al., in preparation).

The amino acid sequence of this protein is: NFMLTQPHSVSESPGKTVTISCTRSSGSIASNYVQWYQQRPGSSPTTVIYEDNQRPSGVPDRFSGSIDSSSNSASLTISGLKTEDEADYYCQSYDSSNHVVFGGGTKLTVL.

Since the original lambda 6-germ line sequence code for a protein containing a proline in position 7, as shown above, and for the sake of preparing the mutants used in this work, three mutants were prepared, one with a single mutation and two with two point mutations each. In this manner, we have used 4 different proteins: the original 6aJL2 and three novel mutants. All novel mutants have a proline at position 8 (H8P). One of them additionally has a phenylaline substituted for leucine at position 2 (F2L-H8P), and the remaining one has the same phenylaline substituted for serine (F2S-H8P).

3. Results and discussion

3.1. Finger printing T. cambridgei venom components

Several aliquots of soluble venom from *T. cambridgei* were applied in amounts of 1 mg protein each, to the HPLC system, separating approximately 60 different components, as shown in Fig. 1A.

For liquid chromatography–mass spectrometry (LC–MS), the off-line system was chosen, because it permits pooling sub-fractions from several columns, when the relative concentration in the whole venom of a given component is low. It also permits further separation before loading into the mass spectrometer. Usually, when a small contaminant is present, the major protein/peptide impairs the proper detection of minor contaminants. The chromatographic profile of this crude venom (Fig. 1A) is similar to others, obtained with venoms from scorpions of the genus *Tityus* [15]. Generally, when the HPLC separation is run for 60 min, the peptides eluting with retention times (RTs) from 8 to 20 min of HPLC are minor components. Most of them correspond to toxins similar to Tc1 [16] or to fragments of other larger peptides, as it will be discussed later. Components eluting with RT from 20 to 32 min correspond to the K^+-channel specific toxins (abbreviated K^+-ScTx). Examples of these are shown in Batista et al. [17]. Among the components eluting with RT of 32–50 min are toxins specific for Na^+-channels

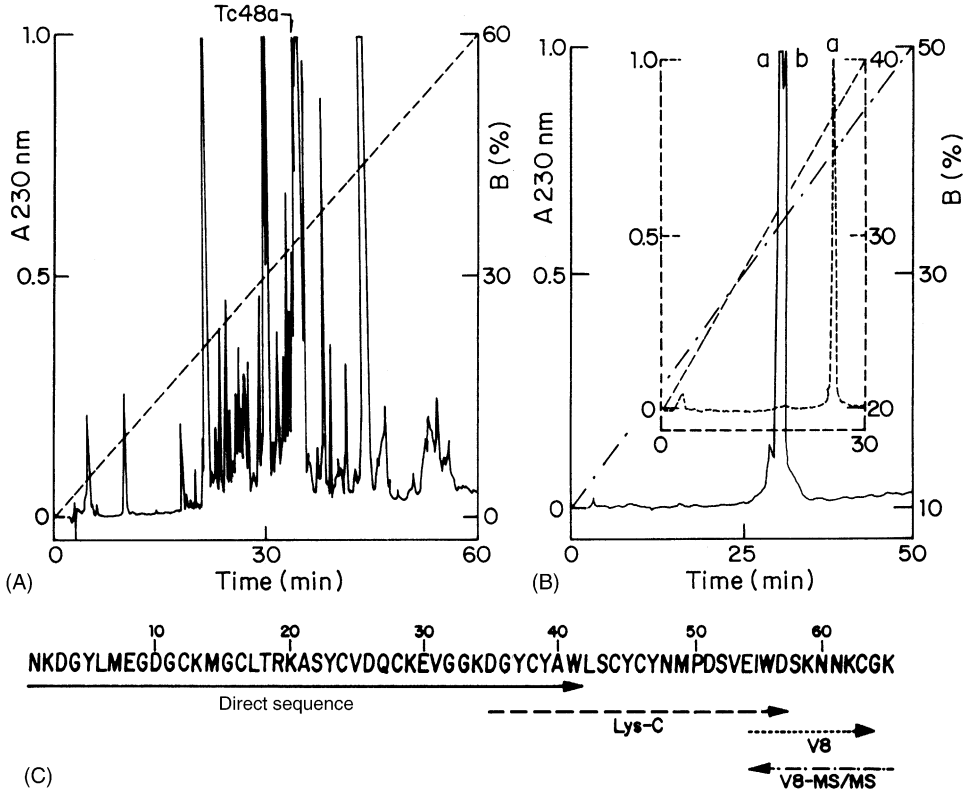

Fig. 1. HPLC separation and amino acid sequence of Tc48a. Panel A: HPLC separation of 1 mg soluble venom from *T. cambridgei* in a C_{18} reversed phase column equilibrated with solution A (water in 0.10% TFA), using a gradient from 0 to 60% solution B (acetonitrile in 0.12% TFA) over 60 min. The elution position of Tc48a is indicated by an arrow at retention time 35.12 min. Panel B: The first plane represents the profile of re-chromatographic separation of the Tc48a fraction in the same analytical column described above using a gradient from 10 to 50% solution B over 50 min (dashes and dots). Two main peaks are labeled as (a) and (b). The second plane of the (B) represents the final HPLC purification of the peak labeled as (a) (Tc48a) using a gradient from 20 to 40% of solution B (dashes) over 50 min (run stopped at 30 min). Panel C: Amino acid sequence of Tc48a determined by direct Edman degradation (residues 1–41), plus peptide obtained after cleavage with endoproteases Lys-C (residues 35–57) and Glu-C (V8) (residues 55–63). Last two residues were determined by collision-induced dissociation mass spectrometry on the same peptide Glu-C (V8) (positions 55–65).

(abbreviated here Na^+-ScTx), and usually are the most important components in terms of relative concentration in the venom [18]. Finally, after RT 50 min are the hydrophobic and higher molecular height components of the venom (this contribution). All the components obtained from Fig. 1A were submitted to spectrometry analysis using both systems mentioned in Section 2. Table 1 summarizes the main molecular masses found when analizing the peptides obtained in Fig. 1A with the LCQ^{DUO} mass spectrometer. The Na^+ and K^+ adducts formed before or during the analysis are not included. Components from seven RTs were not assigned by the ion trap mass spectrometer (labeled with asterisk in Table 1) and were further analyzed by MALDI–TOF. The number of components clearly identified in Table 1 are 102 individual masses. The molecular masses found varies from hundreds of amu to 58 kDa. Table 2 lists all the peptides for which at least the first 10 amino acid residues at the N-terminal amino acid sequence were obtained. Note that four of them were already fully sequenced, as indicated in references [16–18]. It is important to mention that in our previous publication (see [18]), the HPLC semi-preparative column used was run for 90 min. In this communication, the column used was an analytical one, and the time was 0–60 min. For this reason, the RT and the nomenclature used in Tables 1 and 2 do not coincide exactly, but we have decided to keep the initial nomenclature in order to avoid possible confusion on the naming system of the various components. To facilitate reading, in Table 2 we have included a column with all the RT according to Fig. 1A of this communication, after the name given in our original publication (the Tcs followed by numbers), in which the 90 min gradient was used, instead of 60 min of Fig. 1A. The complete amino acid sequence of peptide Tc48a is now described (see below). In summary, the finger print analysis of the soluble venom of *T. cambridgei* indicates that in this venom, there are at least 102 different components, for which 26 have their N-terminal sequence determined; and 5 are now fully sequenced. Not included are several components eluting from the HPLC after the injection artifact, before RT 10 min. These components were not resolved by ESI, but suggested they might be polymers, not necessarily of peptidic nature, now under investigation by our group. It is also conceivable that other components in very minute amounts on the venom, were not analyzed, because they eluted in the

Table 1
Mass finger printing from the total venom of the scorpion *T. cambridgei* by ESI-MS and MALDI–TOF/MS off-line

Retention time	Molecular weight $[M + H]^+$	Retention time	Molecular weight $[M + H]^+$
10.40	268.2	34.78	5757.0; 6927.0
15.05	439.4; 902.3	35.12	7216.0; 7318.3
17.68	487.4	35.55	7385.2
18.75	2446.4	35.98	7152.0; 7405.6
19.36	1983.7	36.51	7073.0
19.87	2551.0; 2665.0	37.18[a]	5912.2; 6943.7; 7126.4; 7938.3; 11021.0
20.72	686.5; 879.5	38.46[a]	7964.5; 8519.86; 10512.0; 10638.6
22.16	4103.1; 4272.0; 4288.0	39.26	7253.2
22.16-2	1310.6; 4303.2	39.26-2	10960.0
23.10	643.5	39.74	10664.0; 14669.0
23.60	1735.6	40.19	7299.0
24.14	4150.3	40.75	6908.8
24.64	3335.9	41.28	6892.9; 10594.0
25.07	3871.8	41.87	10788.0; 11032.0
26.19	4304.4; 4519.0	42.64-1	5504.1; 10847.0
26.86	3521.5; 3742.5	42.64-2	7105.0
27.42	3807.9	42.64-3	6293.0
27.74	662.4; 913.6; 4002.6; 5560.2	43.87[a]	6902.4; 6958.7; 7183.7; 10798.7; 11185.1
28.27	3926.2; 4017.7; 5121.1; 7265.6	45.18	6935.3; 7628.7
29.23	651.4; 1142.5	47.02	44650.0
29.92	2744.1; 3026.6	48.11[a]	6942.4; 11209.0; 14101.6; 26013.2
30.83	7783.0; 8054.0	50.04[a]	6944.7; 10850.0
31.15	7109.4; 7796.4	52.19	57929.0
32.03	743.3; 1026.0; 6929.0	53.90[a]	26324.6; 27058.7
32.75	7266.0	55.58	25323.0
33.52	2757.0	56.64[a]	25621.4; 26917.1
33.95	6944.0	57.23	25402.0; 25519.0; 25604.0
34.35	6032.0		

[a] Molecular weights determined by MALDI–TOF.

valleys of the different chromatogram peaks, but due to the low concentration did not absorb enough to be individually collected, and thus were not included in this analysis.

3.2. Chemical and physiological characterization of toxic component Tc48a

The component labeled Tc48a in Fig. 1A (RT 35.12 min) is a mixture of two peptides with molecular weights of 7216.0 and 7218.3 amu. They were separated by HPLC, as shown Fig. 1B. The front panel shows the profile of the rechromatographic step, where two major components are clearly seen (a) and (b). The back panel (dashed lines), shows the chromatographic separation of the peptide (labeled a), with 7318.3 amu, which was obtained in homogenous form and was sequenced. It was named Tc48a, after its elution pattern on the HPLC column [18]. As it will be shortly described below, Tc48a is a toxin that affects Na^+-channel function. In Fig. 1C, we show the strategy used to fully determine its primary sequence. Direct automatic Edman degradation of native or reduced and alkylated toxin gave unequivocal identification of residues from positions 1 to 41. Lys-C and Glu-C (V8) endopeptidases hydrolysis of the peptide followed by HPLC separation (data not shown) permitted to obtain a series of overlapping segments of peptides that account for the full sequence. The Lys-C peptide eluted at 41:59 min gave sequence from residue 35 to 57, whereas another peptide obtained with V8 protease digestion (eluted at 20:80 min in the HPLC) gave the sequence from residue 55 to 63. The two last residues were obtained by mass spectrometry. The experimentally determined mass of Tc48a (7318.3) was a little bit less than the theoretical molecular mass expected based on the amino acid sequence, which was 7319.3 amu. This suggested the possibility that Tc48a was amidated at the C-terminal residue. In order to verify this finding, a peptide generated by Glu-C digestion (IWDSKNNKCGK) and corresponding to the C-terminus, was isolated and submitted to MS experiments (Fig. 2). The calculated monoisotopic molecular weight of the peptide was $[M + H]^+$ 1292.6 amu and the experimentally determined was $[M + H]^+$ 1291.4 amu with 1.2 amu of difference. Furthermore, this peptide fragmented by collision induced dissociation (CID) showed a (b) ion with 1146.4 amu corresponding to the loss of the amidated lysine residue (molecular mass of 127.0 amu). In this manner we concluded that the lysine in position 65 was amidated.

Earlier experiments conducted with components eluted at RT 35.12 min (labeled Tc48a) in Fig. 1A demonstrated that this fraction contained toxic components when experimentally injected into mice [18]. For this reason it was important to conduct experiments that would indicate in a more controlled physiological condition what was the mechanism of action of Tc48a.

Table 2
N-terminal, full sequences and molecular weight of toxins from *T. cambridgei*

Peptides	Molecular weight	Sequence
Tc1 (18.75)	2446.4	ACGSCRKKCK... [16]
Tc27 (22.16)	4103.1	DEGPKSDCKP...
Tc29 (24.14)	4150.3	FNGAVXIW...
Tc30 (25.07)	3871.8	VFINVKCRGS... [17]
Tc31 (26.19)	4304.4	CSTCLDKP...
Tc32 (26.86)	3521.5	TGPQTTCQAA... [17]
Tc33 (27.42)	3807.9	ILNRCCNDDN...
Tc35 (28.27)	3926.2	TGPQTXXQAA...
Tc37 (28.27)	7265.6	TAIRKCNPRT...
Tc39 (29.92)	2744.1	DDDDLEGFSE...
Tc40 (31.15)	7796.4	IKNGYPRDS...
Tc41 (31.15)	7109.4	KDDYPVDTAK...
Tc43 (32.75)	7266.0	LDGYPLSKNN...
Tc46 (34.35)	6032.0	KEGYLFGSRG...
Tc48a (35.55)	7318.3	NKDGYLMEGD...[a]
Tc48b (35.55)	7385.2	KDGYLVGNDG...
Tc49a (35.98)	7152.0	KDGYLVGNDG...
Tc49b (35.98)	7405.6	KKEGYLVGND... [18]
Tc50 (36.51)	7073.0	LDGYPLSKIN...
Tc54 (39.26)	7253.2	KDGYLMEYGG...
Tc56 (40.19)	7299.0	EKGKEILGKI...
Tc58 (42.64-1)	5504.1	KKFGGFLXXI...
Tc61 (42.64-2)	7105.0	KEGYLLGSRG...
Tc64 (45.18)	7628.7	GLRQKVQSLV...
Tc66 (45.18)	6935.3	SYSGYPVTQK...
Tc83 (57.23)	25402.0	NDQCLVIEIL...

[a] Tc48a full sequence reported in this paper.

The effect of toxin Tc48a was investigated by patch clamping F-11 cells (see Section 2) and recording the inward currents at different test potentials from an holding of −110 mV (see inset of Fig. 3). Fig. 3 shows the recordings of TTX-sensitive currents in control and during the action of different concentrations of Tc48a (0.5, 1.0, 2.1, 4.2, and 8.4 μg/ml). It can be seen that the toxin produced a dose dependent increase in the peak currents and in the current present at times greater than 10 ms after eliciting the test pulse. By analyzing the peak current at 0 mV versus toxin concentration we obtained the dose-response curve shown in the upper right part of Fig. 3 and the concentration for obtaining the resulting half-maximal fractional increase in the peak current was 2.6 ± 0.2 μg/ml ($n = 4$). On the other hand, by observing the decaying portion of the current, it is possible to notice that, at the different toxin concentration, the time constant and the amplitude of the currents is increasing. The time constant of current inactivation at −20 mV, which was 3.36 ± 0.39 ms in control, increased to 7.8 ± 0.3 ms at 8.4 μg/ml of toxin. At 10 ms from the beginning of the test pulse the current is nominally zero in control. Thus, by evaluating this amplitude, at the various concentrations tested, we were able to derive the plot shown at the right lower part of Fig. 3. This type of dose-response curve resulted in a concentration of 0.6 ± 0.05 μg/ml at the half-maximal normalized amplitude value, corresponding to about 82 ± 7 nM concentration. On the whole, it is expected that Tc48a will produce remarkable effects on the physiological response in firing neurons by introducing, at resting potential, a depolarisation caused by the more pronounced window current. Indeed, a general increase in excitability and increased amplitude of the action potential were observed soon after the application of the toxin in large firing F-11 neurons (not shown).

3.3. Discussion on other components from *T. cambridgei* mass finger printing

Tc1 was the first peptide from *T. cambridgei* venom to be completely characterized [16]. It is the shortest known peptide from scorpion venom that recognize K^+-channels. It elutes at RT 18.75 min from the analytical C_{18} column and has a molecular mass of 2446.4 amu. Its three-dimensional structure was recently solved by NMR spectroscopy [19]. The material eluting at RT 22.16 min, denominated Tc27 in Table 2, is a mixture of closely related molecular weight isoforms: 4103.1 (the most intense signal), 4272.0, 4288.0, and 4303.2 amu. The difference between the last three molecular weight values is 16.0 amu interpreted as being the product of oxidation of the peptides. The component with 4103.1 amu was further separated by ion exchange chromatography (data not shown) and its N-terminal amino acid was determined (see Tc27 in Table 2).

The component with RT 25.07 min named Tc30, is a K^+-channel toxin of 37 amino acid residues with molecular weight of 3871.8 amu, shown to be a potent inhibitor of K^+-currents in human T lymphocytes [17].

The peptide labeled Tc31, with molecular mass of 4304.4 amu, had its N-terminal amino acid sequence CSTCLDKP, which is completely different from all the other known scorpion toxins. Its function is unknown. The component eluted at RT 26.86 min has two peptides, from which the molecular mass of 3521.5 amu (most intense signal) corresponds to peptide Tc32, whose full sequence and function has been reported previously [17].

The peptide with N-terminal sequence ILNRCCNDDN presenting two consecutive cysteine residues in positions 5 and 6, has a molecular mass of 3807.9 amu (RT of 27.42 in Table 1). Comparative analysis in data banks showed no significant similarities to any other known protein. Peptides Tc29, Tc35, and Tc37 (Table 2) are peptides for which the function has not been determined.

Tc39 is another interesting peptide with a very acidic N-terminal sequence (DDDDLEGFSE); elutes with RT 29.92 min, and has a molecular mass of 2744.1 amu. It is a completely novel type of sequence in the context of scorpion proteomics, whose function is also completely unknown.

Most peptides eluting between 32 and 50 min in Fig. 1A, as mentioned earlier, are peptides with molecular weights in the order of 7000 amu (see Table 1), and usually correspond to peptides that modify the gating mechanism of Na^+-currents on excitable cell membranes, showing an exquisite species specificity. Some are toxic to mammals, others to invertebrates (crustaceans or insects). The best

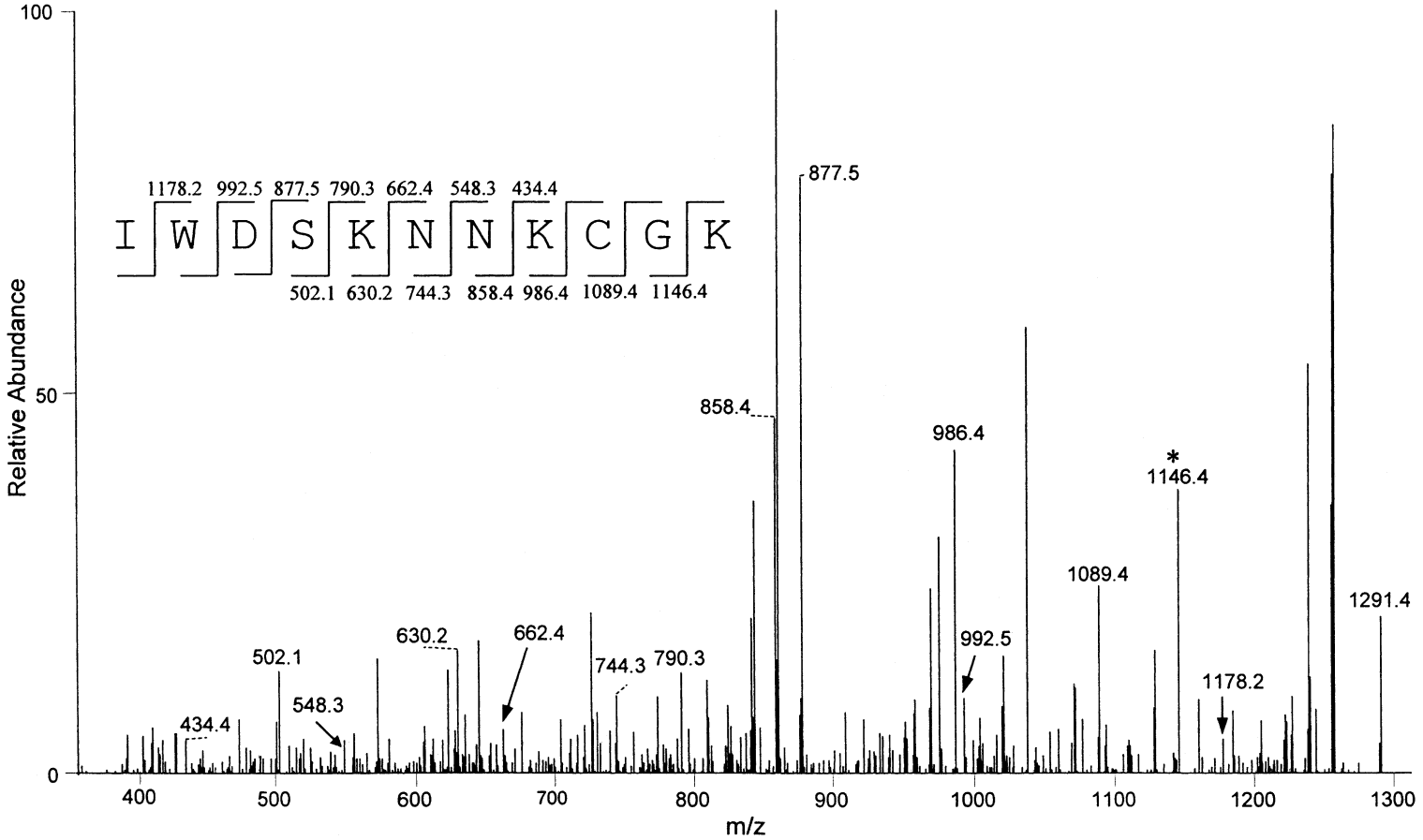

Fig. 2. Determination of the amidated C-terminal peptide. The same endopeptidase V8 digested carboxyl-terminal peptide of Tc48a was subjected to collision-induced dissociation mass spectrum analysis on the $(M + H)^+$ ions at *m*/*z* 1291.4. Fragments of type b- and y-ions are shown above and below the amino acid sequence in the inset of the figure. The b-ion at *m*/*z* 1146.4 represents the loss of the amidated lysine residue labeled with asterisk.

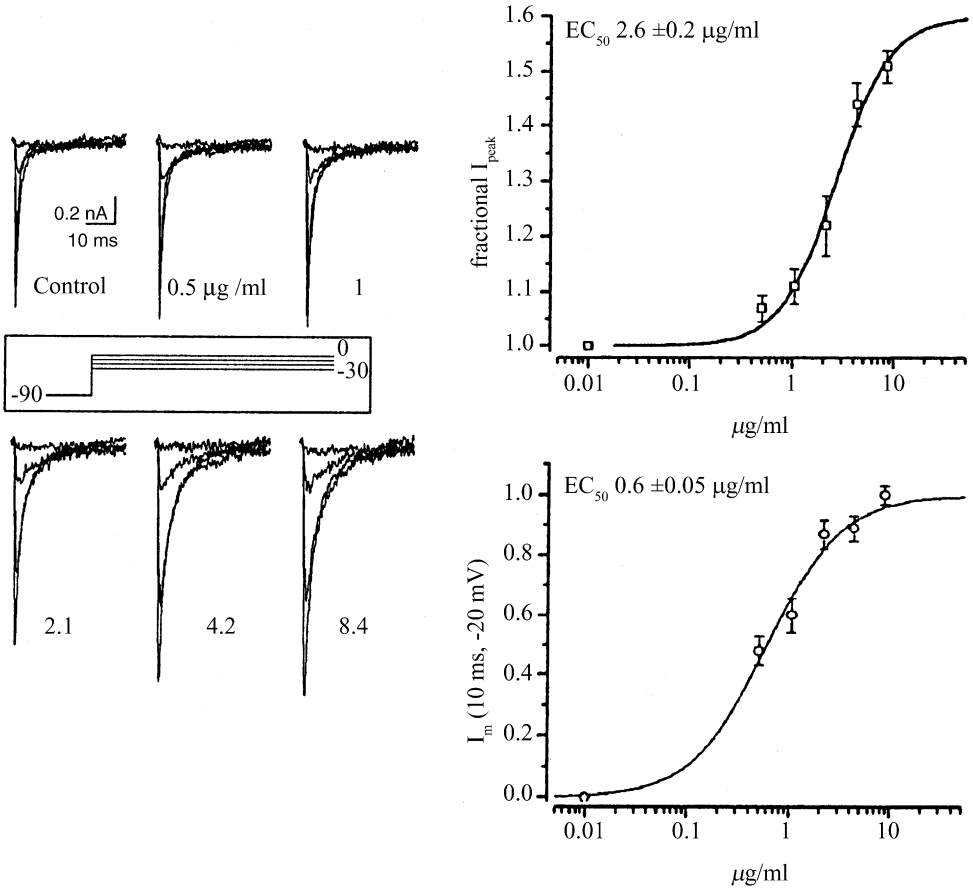

Fig. 3. Electrophysiological recordings of Tc48a effects. Left panels show the superimposed traces of TTX-sensitive sodium currents elicited according to the protocol shown in the inset and recorded in a single F-11 cell in control and during the perfusion of the toxin Tc48a at the indicated concentrations. Right panels show the dose–response curves according to two different criteria: upper) the normalized increase in the peak current elicited at 0 mV; lower) the normalized increase of the amplitude of the current (elicited at −20 mV) at 10 ms after the onset. The theoretical value of the EC_{50} is given in the diagram.

studied are those toxic to mammals. Among these are TC48a, Tc49a, Tc54 and Tc49b [18]. In this communication, as discussed in the preceeding section, we are reporting the complete characterization of Tc48a, which is the second peptide specific for Na^+-channels fully characterized from this venom. Proteins eluting after the 50 min on Fig. 1A are completely unknown, but as shown in Table 1 several of them have molecular weights from 25 to 60 kDa. The N-terminal amino acid sequence of Tc83 (Table 2) is reported here for the first time.

Finally, there are several sub-fractions (labeled with asterisk in Table 1) that were identified using the MALDI–TOF/MS analysis.

3.4. Stability of scorpion toxins under ESI-MS and MALDI–TOF/MS analysis, and determination of the exact number of components

Our experience with scorpion venom finger printing indicates that determination of the exact number of natural compounds produced by the scorpion venomous glands is an open question. Several variables can influence the final results, among which are intrinsic factors, such as possible genetic variations, gender differences [3], age of the scorpions or regional variations due to geographical distribution of the same species, but usually, the extrinsic factors are the most significant ones, among which are: technique used for venom extraction, as recently published in studies conducted with *Parabuthus transvaalicus* [20], time elapsed between extractions, milking of unique specimens or mixing the venom obtained from several animals, time taken for milking hundred of animals, allowing the venom to air-drying or freeze-drying immediately. External factors can modify the final number of different compounds found, like the exposition of the venom to high oxidative atmosphere for hours. Photochemical reactions can occur when the venoms are not light protected. In addition, the presence of endoproteases from cells damaged due to the technique used for extraction: softly (mechanical induced) or abruptly (electrical shock) obtained, can produce cleavage of components. A multistep purification protocol can expose the venom components to artificial modifications, such as oxidation of methionines and tryptophans, deaminations of asparagines and glutamines. This will certainly modify the

retention times in the chromatograms and complicate interpretation of the actual venom composition, at a given time and circumstances. Some of the very low molecular forms, probably are degradation products of larger peptides, either by other venom components (enzymes) or by mass spectrometry conditions. The last situation is supported here by experimental data. It concerns the thermolability of many toxins associated with the presence of proline residues at the N-terminal region of Na^+-ScTx. In fact, collisional thermal dissociation is a well-known process in electrospray ion sources [21–24]. Toxin Tc50 has a proline residue at position 5 in its N-terminal portion (LDGYPLSKIN). To evaluate the thermo stability of this toxin, the electron spray heater capillary was set at 120 and 160 °C. Two aliquots of Tc50

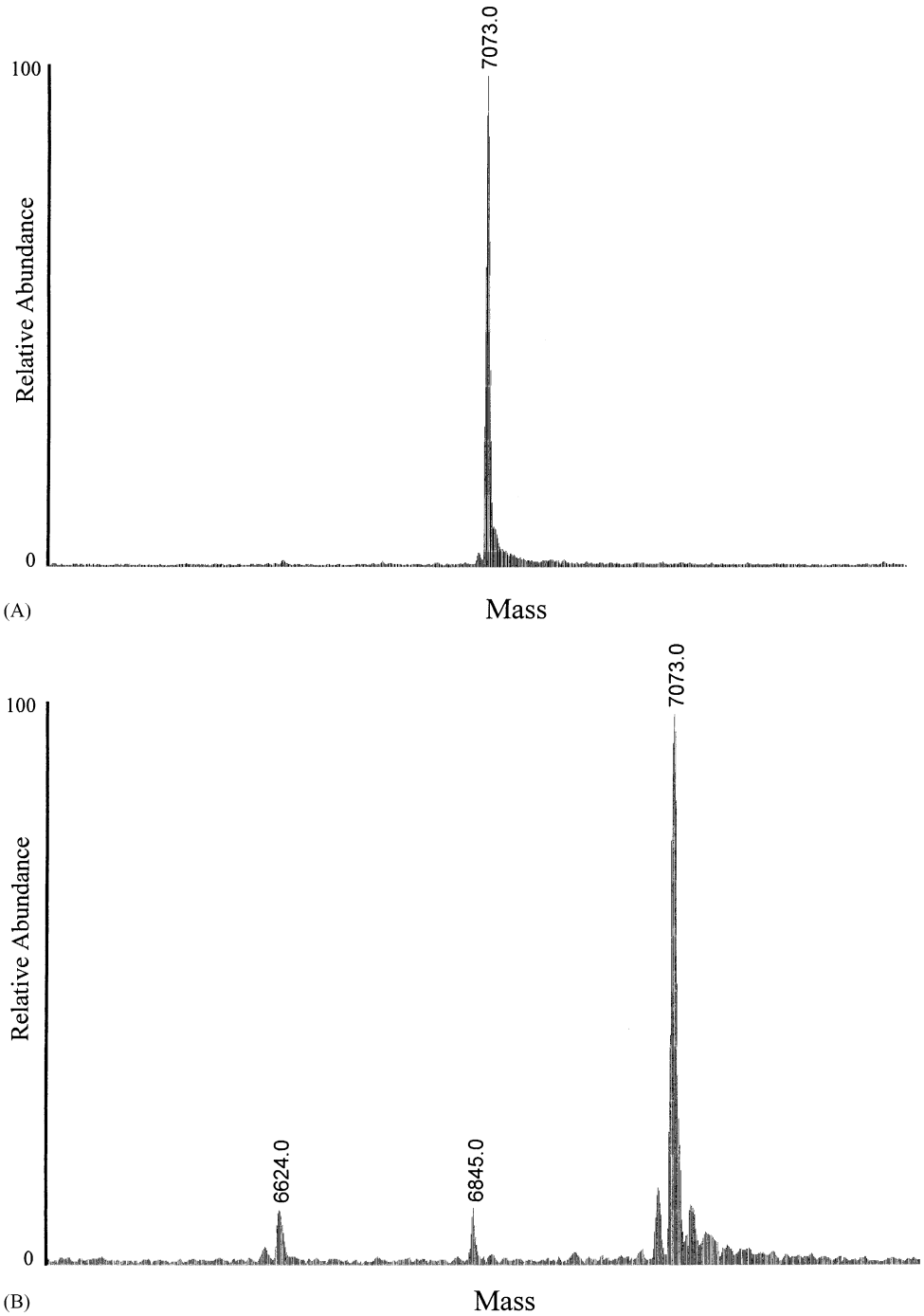

Fig. 4. Thermo-stability of Tc50 under MS analysis. Panel A: molecular weight determination of the toxin Tc50 with the nano-ESI source set at 120 °C. Panel B: deconvoluted mass spectra recorded from toxin Tc50 with the nano-ESI source at 160 °C. The molecular weights at 6624.0 and 6845.0 amu correspond to the thermo-dissociated toxin with loss of the LDGY and LD fragments, respectively.

in homogeneous form were analyzed under these different temperatures. Fig. 4A shows the automatic deconvolution of the spectra obtained at 120 °C from Tc50 presenting just one main peak. The deconvolution of the spectra obtained at 160 °C (Fig. 4B) shows two additional molecular weights, corresponding to the loss of LD and LDGY. The same heat degradation was observed for toxins Tc41, Tc43, and Tc66, which contain a proline residue in its N-terminus. The same phenomenon was observed with toxins from another scorpion *Tityus discrepans* (unpublished results). All these

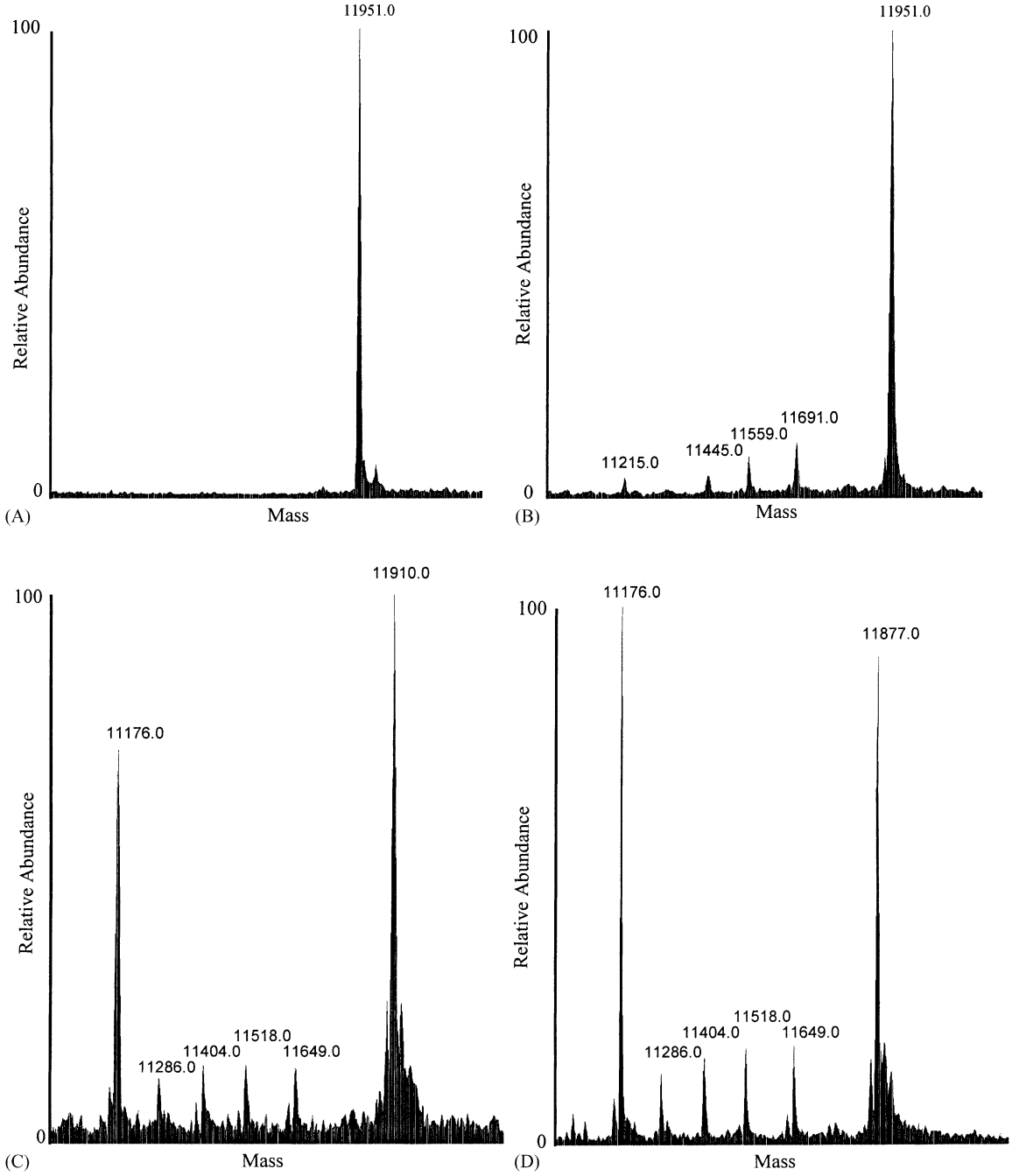

Fig. 5. Mass spectrometry analysis of mutants of immunoglobulin 6aJL2. *In source* thermo-dissociation mass spectrometry analysis of the mutants of the variable regions of the immunoglobulin 6aJL2 was performed at two different temperatures. Panel A: deconvoluted mass spectra from the native 6aJL2 recorded with the ion source set at 100 °C. Panel B: deconvoluted mass spectra from the native 6aJL2 at 160 °C showing different levels of dissociation. Panel C: deconvoluted mass spectra of the double point mutant F2L-H8P obtained with the ion source at 160 °C. The molecular weight 11176.0 amu represents the total loss of the N-terminal segment NLMLTQ located preceding the first proline at position 7. Panel D: deconvoluted mass spectra of the mutant F2S-H8P performed with the ion source set at 160 °C showing high level of dissociation. The molecular weights 11649.0, 11518.0, 11404.0, 11286.0 and 11176.0 represent the loss of amino acid fragments NS, NSM, NSML, NSMLT and NSMLTQ, respectively.

toxins have free N-terminal segments, not holded in place by adjacent cysteine residues involved in disulfide bridge formation. In order to study if this was a particular case of scorpion toxins, we decided to use a model protein and three site-directed mutants prepared from the protein 6aJL2, a V_L domain carrying lambda 6-germ line sequence. The amino acid sequence of native protein 6aJL2 and the positions where the amino acid residues were changed are described in Section 2. Fig. 5A shows the automatic deconvolution of the native 6aJL2 protein with the capillary heater at 100 °C. At this temperature the molecular weights observed was 11951.0, corresponding to native 6aJL2. The same sample analysed at 160 °C (Fig. 5B) shows several additional peaks coming from the degradation of the N-terminal portion of the protein. Although the additional degradation components seen in this Fig. 5B are relatively small, they increase by a factor of four the number of total components detected. For the other two mutants F2L-H8P and F2S-H8P, when analyzed at 100 °C there is only one molecular mass detected for each one of them (data not shown). However, when they are analyzed at 160 °C five additional molecular masses are detected (Fig. 5C and D), which correspond to the fragmentation at the level of the mutated proline in position 7 towards the amino acids situated in positions 6, 5, 4, and 3. The main component in both spectra have the expected molecular mass for the intact mutants (11910.0 and 11877.0 amu). The fragmentation due to the presence of the double proline (positions 7 and 8), make the N-terminal region more liable, splitting the peptide bounds between amino acids at positions 2–3 (11649.0), 3–4 (11518.0), 4–5 (11404.0), 5–6 (11286.0), 6–7 (11176.0), as shown in the figure.

From these experiments two important points are clear: first, the degradation process starts before the proline at position 7 and a synergistic effect is observed when two prolines are in adjacent positions (7 and 8). It is worth noting that the peptide bonds between positions 1 and 2 were not susceptible to fragmentation. The mutants at position 2 (F/L and F/S) were prepared in order to verify if it was not a special case, depending on the type of amino acid occupying position 2. At least with these two mutants it seems to be independent of the amino acid in position 2.

Furthermore, toxin Tc50, the protein 6aJL2 and its three different mutants were also analyzed by MALDI–TOF/MS. The samples were co-crystallized with α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid and ionized by high laser intensity (50–100% arbitrary units). The results showed absolutely no degradation of the samples when analyzed with sinapinic acid and DHB. However, when the analysis was performed by CHCA co-crystallization, different levels of degradation were observed. Tc50 showed two additional low intensity peaks corresponding to the loss of LD and LDGY, exactly the same when analyzed by ESI-MS at 160 °C. Unexpected results were observed with 6aJL2 and mutants that do not showed any degradation in CHCA, but when the matrix peaks range (200–1000 amu) was set, additional peaks were observed at 809.40 and 1201.50 *m/z* for H8P, F2L-H8P and F2S-H8P mutants.

These results clearly indicate that the degradation phenomenon depends on the matrix used, consistent with the observation that CHCA causes more analyte fragmentation than DHB and that the analyte internal energy is dependent on the exothermicity of the proton-transfer reaction. The classification of the matrices as hot or cold was dependent on their propensity to produce fragmentation of glycoproteins [25,26]. Another possible explanation, specially in the case of scorpion toxins that have proline residues in their N-terminus, is simply the temperature at which the matrices sublime [27]. Proline plays an important role in the stability of toxins under ESI-MS and MALDI–TOF/MS analysis when located at the N-terminal segment not holded in place by disulfide bridges. The same “proline effect” (cleavage at the N-terminal side of proline), is a well-known phenomenon in experiments of collision-induced dissociation (CID) of peptides [28–30]. Fragmentation of whole proteins using high charge state ions (12^+–5^+) by ion trap collisional activation revealed fragments preferentially at the N-terminal sides of proline residues and the C-terminal sides of acidic residues [31].

Having in mind the thermal collisional dissociation in electrospray ion sources, the same HPLC peaks used to perform the mass finger printing of the *T. cambridgei* venom at 110 °C (Table 1) were used to repeat the experiment setting the heater capillary at 160 °C. Surprisingly, we were able to detect the double number of molecular weights in the same venom (data not shown).

Acknowledgements

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