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Rapid Purification and Molecular Modeling of AaIT Peptides from Venom of *Androctonus australis*

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As recombinant viruses expressing scorpion toxins are moving closer toward the market, it is important to obtain large amounts of pure toxin for biochemical characterization and the evaluation of biological activity in nontarget organisms. In the past, we purified a large amount of *Androctonus australis* anti-insect toxin (AaIT) present in the venom of *A. australis* with an analytical reversed-phase column by repeated runs of crude sample. We now report 20 times improved efficiency and speed of the purification by employing a preparative reversed-phase column. In just two consecutive HPLC steps, almost 1 mg of AaIT was obtained from 70 mg crude venom. Furthermore, additional AaIT was obtained from side fractions in a second HPLC run. Recently discovered insect selective toxin, AaIT5, was isolated simultaneously from the same venom batch. It shows different biological toxicity symptoms than the

Abbreviations used: AaIT, *Androctonus australis* anti-insect toxin; AaH1, AaH2, AaH3, AaH4, *Androctonus australis* alpha anti-mammal toxin; BCA, bicinchoninic acid; BjIT2, *Buthotus judaicus* depressant anti-insect toxin; Css2, *Centruroides suffusus suffusus* beta anti-mammal toxin; CsEV3, variant 3 from *Centruroides sculpturatus* Ewing; 3-D, three-dimensional; ESI-MS, electrospray ionization mass spectrometry; HFBA, heptafluorobutyric acid; LqhIT2, *Leiurus quinquestriatus hebraeus* depressant anti-insect toxin; LqhαIT, *Leiurus quinquestriatus hebraeus* alpha anti-insect toxin; Lqq3, *Leiurus quinquestriatus quinquestriatus* anti-insect and anti-mammal toxin; Lqq5, *Leiurus quinquestriatus quinquestriatus* alpha anti-mammal toxin; LqqIT1, *Leiurus quinquestriatus quinquestriatus* excitatory anti-insect toxin; LqqIT2, *Leiurus quinquestriatus quinquestriatus* depressant anti-insect toxin; MALDI-TOF-MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MS, mass spectrometry; PDB, protein data bank; SCR, structurally conserved regions; TFA:, trifluoroacetic acid; Ts7, *Tityus serrulatus* beta anti-mammal toxin.

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known excitatory and depressant insect toxins. AaIT5 gave 100% mortality with a dose of less than 1.3 μg against fourth-instar tobacco budworms *Heliothis virescens* 24 h after injection. During the purification process, we implemented mass spectrometry in addition to bioassays to monitor the presence of AaIT and AaIT5 in the HPLC fractions. Mass spectrometric screening can unambiguously follow the purification process and can greatly facilitate and expedite the downstream purification of AaIT and AaIT5 eliminating the number of bioassays required. Further, electrospray ionization was compared with matrix-assisted desorption/ionization and evaluated as a method of choice for mass spectrometric characterization of fractions from the venom purification for it provided higher mass accuracy and relative quantitation capability. Molecular models were built for AaIT5, excitatory toxin AaIT4, and depressant toxin LqhIT2. Three-dimensional structure of AaIT5 was compared with structures of the other two toxins, suggesting that AaIT5 is similar to depressant toxins. Arch. Insect Biochem. Physiol. 38:53–65, 1998.

Key words: AaIT; AaIT5; mass spectrometry; *Heliothis virescens*; HPLC; scorpion toxin; molecular modeling

INTRODUCTION

A number of peptide anti-mammal and anti-insect toxins have been purified from the venom of various scorpion species and their primary sequences have been determined (Rochat et al., 1979; Zlotkin et al., 1991; Bontems et al., 1991; Crest et al., 1992; Debin et al., 1993; Becerril et al., 1995; Nakagawa et al., 1997). For several toxins, their three-dimensional (3-D) structures were determined by X-ray diffraction (Housset et al., 1994; Zhao et al., 1992) and nuclear magnetic resonance (NMR) (Bontems et al., 1991; Johnson and Sugg, 1992; Inisan et al., 1995; Landon et al., 1997), and it has been accepted that the integrity of the 3-D structure of peptides is essential for their interaction with ionic channels (Sabatier et al., 1987). The relationship between 3-D structure and the interaction with ion channel is carefully discussed (Sabatier et al, 1993; Inisan et al, 1995; Landon et al., 1997). These scorpion toxins affect the sodium, potassium, chloride, and calcium channels. Generally long chain (60–70 amino acids) peptides mainly have an effect on sodium channels (Darbon et al., 1982; Fontecilla-Camps et al., 1988; Fontecilla-Camps, 1989; Loret et al., 1990), and short chain (less than 40 amino acids) mainly on the potassium channels (Chicchi et al., 1988; Zerrouk et al., 1993; Legros et al., 1996). Chlorotoxin has been demonstrated to act on reconstituted rat epithelial chloride channels from embryonic rat brain preparations (Debin et al., 1993). Anti-mammal toxins affecting sodium channels are subclassified into α- and β-toxins (Jover et al., 1980; Couraud et al., 1982), and anti-insect toxins into excitatory, depressant, and α-insect toxins (Zlotkin et al., 1985, 1995). The venom of the scorpion *Androctonus australis* has been extensively studied, and the sequences of 11 toxins, including both mammal and insect toxins, have been determined (Rochat et al., 1972, Kopeyan et al., 1979; Martin et al, 1987; Darbon et al., 1982; Loret et al., 1990, 1991; Bougis et al., 1989; Mansuelle et al., 1992, Nakagawa et al., 1997).

Among the insect selective toxins, AaIT obtained from the venom of *A. australis* is the most toxic peptide to some insects so far reported (Zlotkin et al., 1971, 1994, 1995). It induces fast contraction paralysis at a very small dose. Its toxicity to lepidopterous larvae is lower than its toxicity to dipterous and coleopterous insects (Herrmann et al., 1990, 1995). Because AaIT has high potency and strict insect selectivity, the gene coding of this toxin was introduced into baculoviruses to be used as a biopesticide for lepidopterous insects (Maeda et al., 1991; Stewart et al, 1991; McCutchen et al., 1991, McCutchen and Hammock, 1994). Baculoviruses expressing the toxin showed an increased speed of kill with the tobacco budworm *Heliothis virescens* (McCutchen et al., 1991; McCutchen and Hammock, 1994), which results in a substantial reduction of feed-

ing damage (Hoover et al., 1995). We recently found a new insect toxin, AaIT5, in the venom of *A. australis* (Nakagawa et al., 1997). This toxin manifests stronger toxicity against tobacco budworm, as compared with AaIT, and it may be an improved tool for use in recombinant pest control agents. As recombinant viruses expressing scorpion toxins move toward the market, it is important to obtain large amounts of highly pure toxin for evaluation of toxicity in nontarget organisms and their biochemical characterization including the primary structure. Thus, it is critical to have rapid and high yield purification procedures.

We describe a significantly improved and more rapid purification procedure for AaIT and AaIT5 over the one reported previously (Nakagawa et al., 1997). We used a preparative reverse-phase high-performance liquid chromatography (RP-HPLC) column in the first purification step and an analytical and microbore column for the downstream purification. The presence of the peptide toxins was monitored by mass spectrometry (MS) and bioassay against tobacco budworms in parallel to show that MS approach has a potential to minimize the number of bioassays needed in the purification process. Also electrospray ionization (ESI) was evaluated with respect to matrix assisted laser desorption/ionization (MALDI) and selected as a preferred ionization technique to monitor the presence of peptide toxins by MS. 3-D models were built for LqhIT2, AaIT4, and AaIT5 using molecular modeling technique based on a known structure for variant 3, and the structures were compared.

MATERIALS AND METHODS

Purification of Toxins

The crude venom (70.8 mg) of *A. australis* (Sigma Chemical Co., St. Louis, MO) was homogenized in 5 ml of ice-cold extraction buffer (1.0 M acetic acid with 20 mM H_2SO_4) using a Potter-Elvehjam homogenizer. The material remaining in the homogenizer was washed with another 5 ml of extraction buffer. The combined homogenates were centrifuged for 10 min at 10,000 rpm (Sorval RC-5B, rotor SS-34). The pellet was re-extracted as described above, and the supernatants were combined. HPLC purifications were performed with a Perkin Elmer Series 410 pump and a Perkin Elmer Diode Array Detector. Samples were loaded through a Rheodyne injector with a 5 ml loop. The system was controlled by Perkin Elmer software Omega-4 running on a PC computer (Everex 386/20). Fractions were collected manually according to the absorption at 280 nm.

For the first HPLC purification, the combined supernatant (20 ml) was loaded repeatedly through 5-ml loop injector onto a preparative reversed-phase C4 column (22 × 250 mm; Vydac, Hesperia, CA) at a flow rate of 10 ml/min. The gradient was 0–60% CH_3CN/0.1% trifluoroacetic acid (TFA) in 60 min. For the second purification, the fraction of interest was diluted with 0.1% heptafluorobutyric acid (HFBA)/water to lower the CH_3CN concentration. The second step was performed using an analytical reversed phase Vydac C4 column (4.6 × 250 mm) at a flow rate of 0.6 ml/min; 5-ml aliquots of samples were injected repeatedly to load the whole sample before starting the gradient. The gradient was 0–20% CH_3CN/0.1% HFBA in 10 min, 20–50% CH_3CN/0.1% HFBA in 60 min, and 50–60% CH_3CN/0.1% HFBA in 5 min. For purification of AaIT5, a microbore reversed phase column (1.0 × 150 mm; PLRP-S 300A, Michrom Bioresources, Auburn, CA) was used with a flow rate of 0.05 ml/min. This HPLC run was performed on a Ultrafast Microprotein Analyzer (Michrom Bioresources, Auburn, CA). The gradient was 0–60% CH_3CN/0.1% HFBA in 120 min.

Mass Spectrometric Analysis

The HPLC fractions were screened by positive ESI-MS for the presence of AaIT and AaIT5 on a triple quadrupole VG Quattro-BQ mass spectrometer (VG Biotech, Altrincham, UK). Spectra were acquired over a range of m/z 200–2,000 at 20 s/scan, and the raw data were processed by built in "MaxEnt" software (Ferrige et al., 1991), which transforms the signal from series of multiply charged ions into single peaks corresponding to the individual components present in the sample preserving their relative abundancies (e.g., see Fig. 6A). A total of 10 μl of sample aliquots in 50% CH_3CN were injected using a Rheodyne injector, and a 5-μl/min flow of CH_3CN/1% formic acid delivered the sample into the source.

Comparison of ESI and MALDI, two common ionization techniques used in bioanalytical MS, was performed on the above-mentioned quadrupole mass spectrometer and on a linear MALDI-TOF-MS (Hewlett Packard G2030A). Two purified scorpion toxins—chlorotoxin (average MW 3996.8) and LqhIT2 (average MW 6758.6)—were used as standard and analyte, respectively. A set of mixtures

with varying ratios of LqhIT2/chlorotoxin was prepared and analyzed both by ESI-MS and MALDI-TOF-MS. Samples for ESI-MS were dissolved in 50% CH_3CN. Samples for MALDI-TOF-MS were prepared by dried droplet method or by depositing aqueous mixture on the matrix crystallized on the probe from organic solvent (Vorm et al., 1994).

Molecular Modeling of AaIT5 and Related Toxin

All modeling was performed on a Silicon Graphics workstation using the software InsightII (Version 95.0) from Molecular Simulations. Homology software was used for model generation, and Discover software for energy minimization using the Consistent Valence Force Field (CVFF). The sequence of interest was aligned to variant 3, which is registered as 2SN3 in Protein Data Bank (PDB) (Zhao et al., 1992).

Bioassay

An aliquot of each fraction was concentrated by vacuum centrifugation (Speed Vac, ATR, Laurel, MD), the volume of aliquot is shown in the corresponding table. After re-dissolving each concentrated fraction in distilled water, 2–3 μl of each sample was injected into late fourth-instar larvae of tobacco budworms just before molting. For the AaIT5 containing fraction collected from the microbore HPLC the protein concentration was estimated by bicinchonic acid (BCA) (Pierce Chemical Co., Rockford, IL) protein assay (Smith et al., 1985), with bovine serum albumin (BSA) as the standard. The symptoms in the larvae were observed at 24 h after injection, and the lethal effect (+) was recorded. The toxic effect in which larvae were paralyzed but not dead at 24 h was indicated by a ± sign. The number of symbols indicates the number of larvae used.

RESULTS AND DISCUSSION

Purification

The elution profile from the first HPLC run with the preparative column is shown in Figure 1. The toxicity data and major mass peaks obtained by ESI-MS analysis of four fractions are shown in Table 1. Fraction III was considered to be the one containing AaIT, because it had a major mass peak of 7823 in ESI-MS data (Fig. 2), corresponding to the expected mass of AaIT, and it also caused very strong paralytic effect on tobacco budworms (Table 1). The ESI spectrum indicates the presence of AaH1 (6803) in fraction III in fairly large quantities, but this peptide was characterized earlier as the anti-mammal toxin, hence is not of interest in this study. We assayed all remaining fractions, including I, II, IV, as well, but none was highly active against tobacco budworms.

After dilution of fraction III (15 ml) with 35 ml of 0.1% HFBA/water, it was further separated using an analytical C4 column eluted with CH_3CN/0.1%HFBA (Fig. 3). The toxicity against tobacco budworms was recognized in fraction 7 as well as in fractions 4 and 5 (Table 2). Fraction 7 contained mainly AaIT as shown by a single peak at mass of 7823 (Fig. 4). The whole fraction was lyophilized to yield 700 μg of AaIT. In order to get more AaIT, the two adjacent fractions were rerun under identical conditions. This way an additional 200 μg of AaIT was recovered (data not shown).

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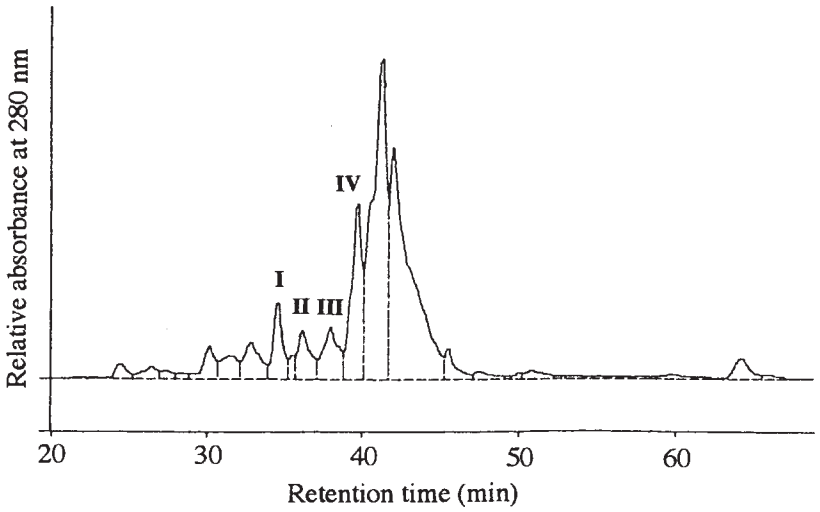


Fig. 1. First HPLC purification step of crude venom using a preparative Vydac C4 column (22 × 250 mm). Sample was eluted with an CH_3CN/0.1% TFA gradient.

TABLE 1. Biological Activity and Predominant Mass Numbers of Fractions in First HPLC Separation

Faction[a]	Volume of fractions (ml)	Activity[b]	Major mass in ESI-MS
I	17	---	7241
II	14	---	7441
III	15	+±-	7823
IV	15	±--	6851

[a]See the peak assignments in Fig. 1.
[b]0.3–0.4 ml was taken from each fraction and dried by vacuum centrifuge, then dissolved in 50 μl of water for bioassay. A total of 3 μl was injected into fourth-instar tobacco budworms and the symptoms were observed; +, dead after 24 hr; ±, paralyzed but not killed at 24 hr; -, no effect.

HPLC step (Fig. 3) were toxic to tobacco budworms, they were further purified. They were combined and diluted three times with 0.1% HFBA/water and again run as described for the second HPLC step (Fig. 5A). Only fraction c was active against tobacco budworms. The ESI-MS of this fraction indicated presence of multiple components. Therefore fraction c was further purified by microbore column HPLC (Fig. 5B). Only fraction c-3 was toxic to tobacco budworms (Table 3). Processed ESI-MS data show that a component of molecular weight 6882 was predominant in fraction c-3. This peak was not detected in other fractions c-1, 2, and 4. The mass 6882 corresponds to the scorpion anti-insect toxin AaIT5 as reported elsewhere (Nakagawa et al., 1997).

Previous purification of AaIT using an analytical Vydac C4 column (Nakagawa et al., 1997) was much more labor intensive and time consuming. In the first HPLC step, only 7 mg venom could be loaded on the column (unreported data); 91 HPLC runs were required to separate the crude venom. Employment of a preparative RP-HPLC column in the initial step, instead of an analytical column, greatly facilitated our toxin purification process. Since the acetonitrile concentration for the elution of AaIT was 50%, it was sufficient to dilute the fractions from the preparative run approximately three times. No lyophilization step was necessary during the purification. This approach saved significant time and permitted direct injection of the diluted fractions on the second HPLC column at increased flow rate. The total time required for the purification of AaIT was cut down by 95% compared with the previous procedure.

In the original purification, 1.4% of AaIT was obtained from the crude venom (Nakagawa et al., 1997). Total amount of AaIT obtained by the new HPLC purification was about 900 μg (1.3%). The venoms were from different batches and the proportion of AaIT might have been slightly different in each, but one can assume that the described method allowed to isolate toxin with a similar or better yield than in the previous study as at present the consumption of material was higher due to the verification of new analytical screening by MS.

MS monitoring was able to trace molecular weight of AaIT after the first HPLC purification step (Fig. 2), even though there were a number of components in the sample. Spectra also indicate increasing purity of the toxin throughout the isolation steps. ESI-MS is a powerful tool to monitor the compound of interest, because of its speed, sensitivity, and accuracy as shown below. By con-

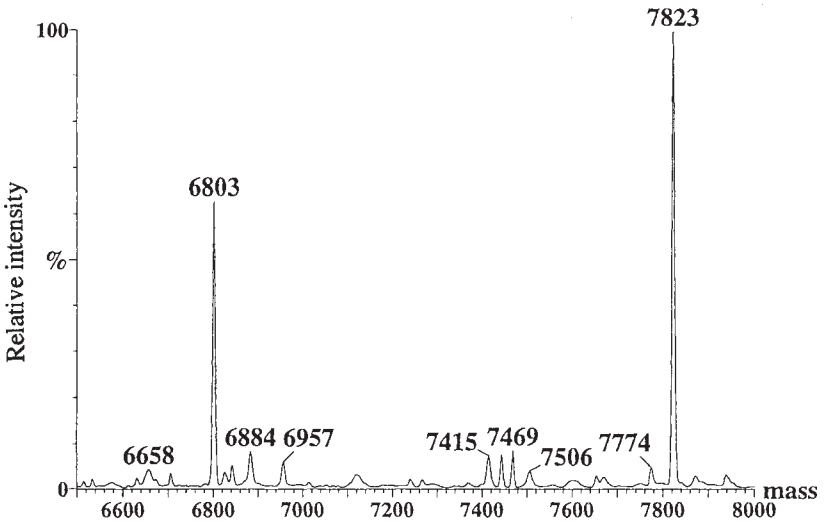


Fig. 2. Mass spectrum obtained from ESI-MS analysis of fraction III in Fig. 1.

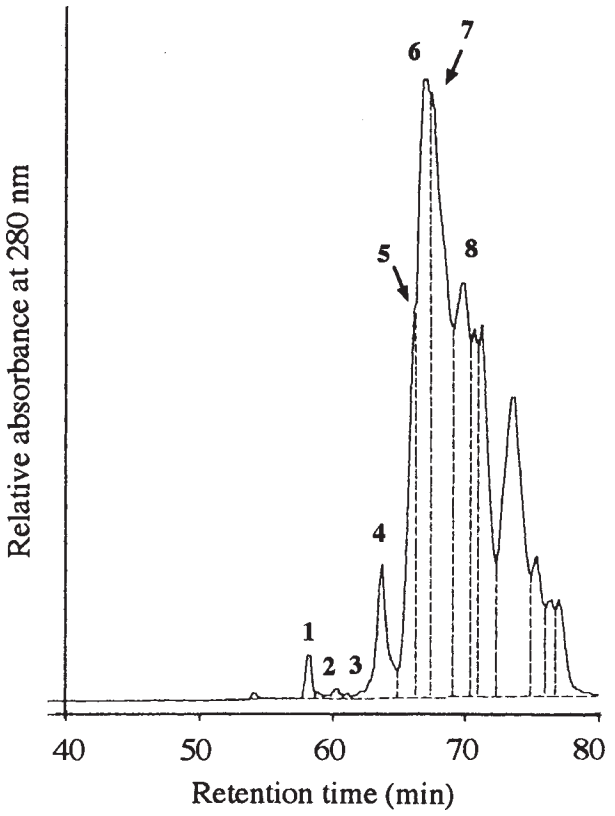


Fig. 3. Second HPLC purification step of fraction III from first HPLC step (Fig. 1). The sample was loaded on an analytical Vydac C4 column (4.6 × 250 mm) and eluted with a gradient of CH_3CN/0.1% HFBA.

trast, microgram quantities of material are needed for bioassays and the material is sometimes used up before completion of the purification. MS screening permits reduction of the number of bioassays, resulting in a higher yield of pure toxin, faster completion of the purification process, and lower material expenses. MS data also provide a good basis for a future search for new uncharacterized toxins and for comparison of venom composition variation between species.

TABLE 2. Biological Activity of the Fractions Collected from the Second HPLC Separation

Faction[a]	Volume of fractions (ml)	Volume of aliquots[b] (µl)	Activity[c]
1	0.8	40	--
2	0.5	25	--
3	0.3	15	--
4	4.0	200	±-
5	1.0	50	±±
6	0.5	25	--
7	1.0	50	++
8	0.7	70	--

[a]For peak assignments see Fig. 3.
[b]The volume of aliquots taken from each fraction and dried by vacuum centrifuge, then dissolved in 50 µl of water for bioassay. A total of 3 µl was fourth-instar botacco budworms.
[c]See Table 1.

Biological Activity

The new purification procedure allowed to isolate sufficient amount of AaIT5 to characterize its biological activity and complement the sequence information that was established in previous work (Nakagawa et al., 1997). The scorpion toxins known so far were not very toxic to lepidopterous insects. For example, the 50% lethal dose of AaIT to kill the lepidopterous pest larvae *Spodoptera littoralis* 6 days after injection was 1.4 µg/100 mg body weight (Herrmann et al., 1990). As shown in Table 3, fraction c-3 containing AaIT5 was extremely toxic to tobacco budworm, resulting in 100% 24-hr postinjection mortality of 1.3 µg/100 mg. The new toxin AaIT5 did not show any paralytic effect on mice at the dose of 6 µg/15 g body weight, whereas the other fraction in the same venom corresponding to AaH2 as reported (Nakagawa et al., 1997) was extremely toxic to mice (100% mortality at 0.75 µg/15 g). This interesting spectrum of biological activity and potency classifies AaIT5 toxin among the promising candidates for pest control applications.

Comparison of ESI-MS and MALDI-TOF-MS

For the molecular weight determination at the current status of instrumentation available at UC Davis the ESI-MS was a preferred technique based on the comparison study. The main issues that needed to be addressed for the monitoring purposes were the accuracy in mass determination and capability for constant relative response for toxins present in mixtures. Two ionization methods ESI and MALDI were compared for mass accuracy, speed of analysis, limit of detection, quantitation and sample consumption. Table 4 summarizes the results of the comparison. Figure 6A,B shows an example of ESI and MALDI-TOF mass spectra. In ESI-MS, the signal for one peptide was divided among multiply charged peaks (left), which worsened somewhat the limit of detection; by contrast, it improved mass accuracy as each peak provides an independent measurement of the peptide mass. This decreased ambiguity of toxin assignment. The software MaxEnt allowed us to find the true mo-

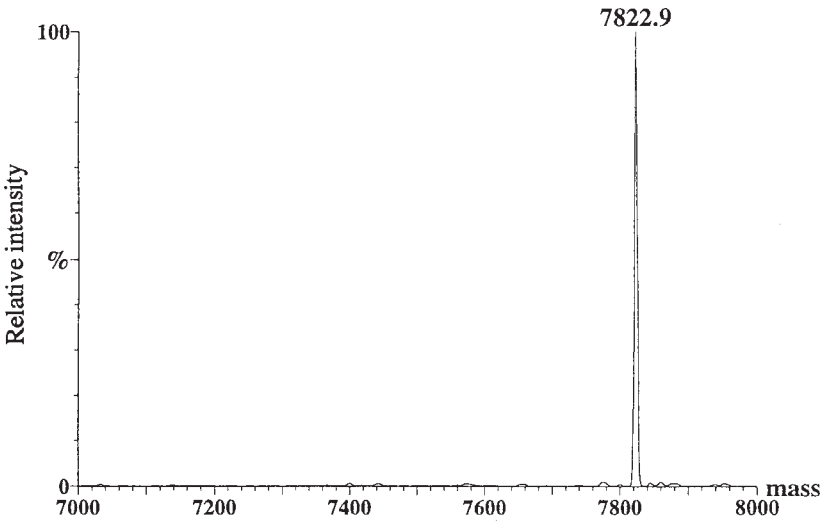


Fig. 4. Mass spectrum obtained from ESI-MS analysis of fraction 7 in Fig. 3.

lecular weights of the most significant components from the raw data (righthand side of Fig. 6A) with a preserved relative abundance. By contrast, MALDI-TOF-MS offered a single major peak for each component and thus provided higher sensitivity and lower sample consumption (Fig. 6B). The analysis suffered by lower mass accuracy and larger variability in signal caused by irreproducibility of the sample-matrix deposition. Sensitivity of MALDI-TOF-MS was increased by raising the laser power sacrificing mass accuracy as the peaks were broadened by adducts formation. Figure 7 shows a correlation of the signal ratio to the concentration ratio. ESI-MS provided a linear dependence and is therefore more suitable for quantitative comparisons while MALDI-TOF-MS data showed large variability depending on the crystallization of the sample on the probe. The MALDI-TOF signal of the LqhIT2 was poor for all molar ratios. A spectrum of individually deposited toxin had a better signal-to-noise (SN) ratio, suggesting that a suppression due to a sample co-crystallization can explain the observed behavior. ESI-MS spectra represented in a more accurate fashion the status of purity in HPLC fractions and helped avoid sensitivity to sample preparation prior to the analysis observed in MALDI.

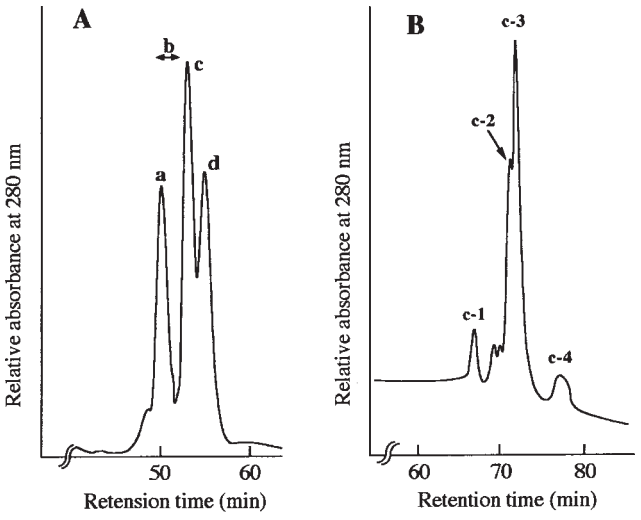


Fig. 5. **A:** HPLC on analytical C4 Vydac column (4.6 × 250 mm) of the combination of fractions 4 and 5 from second HPLC step (Fig. 3). **B:** Microbore C18 column (1.0 × 150 mm) of fraction c from third HPLC step (A).

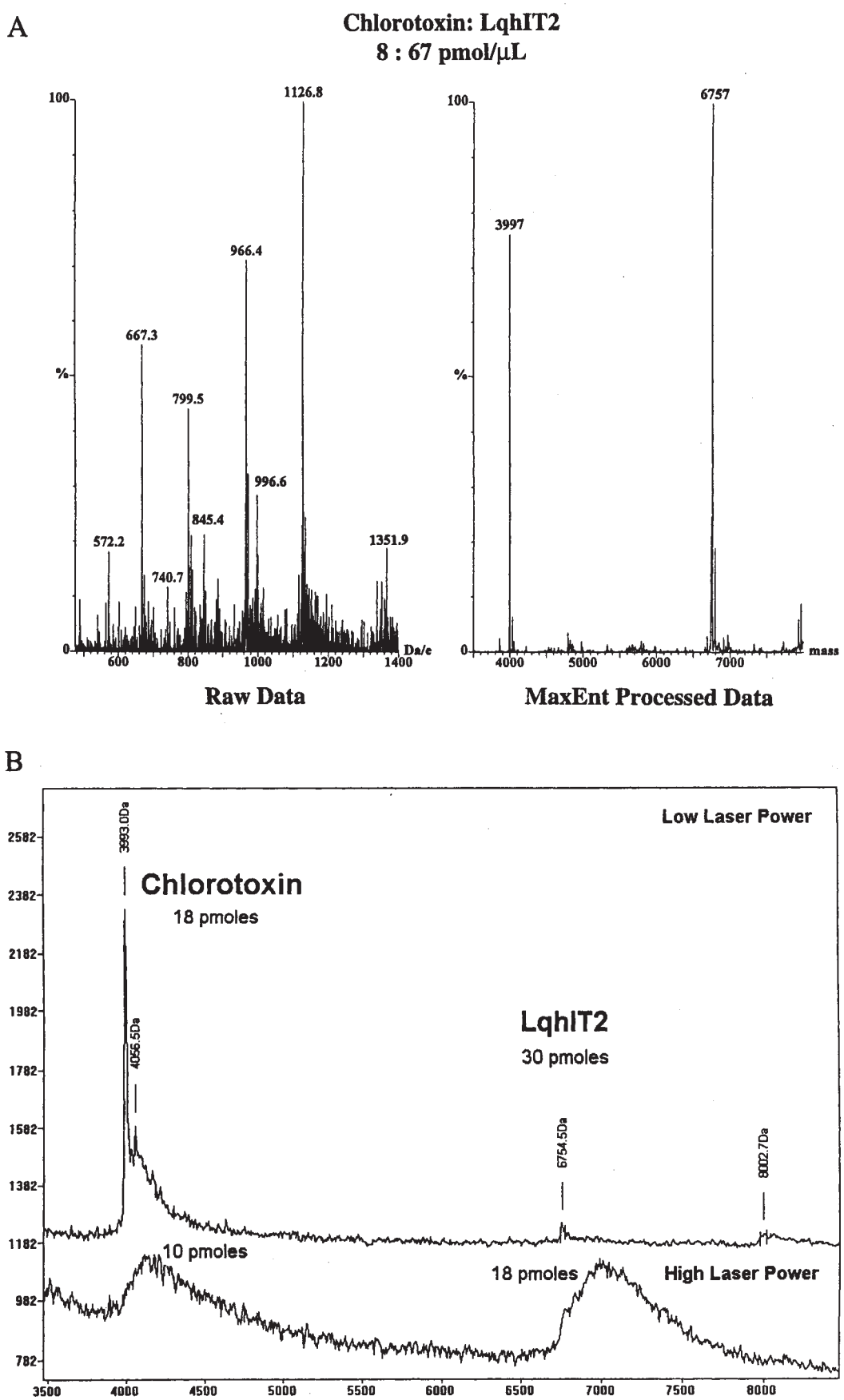
TABLE 3. Biological Activity of the Various Fractions Collected from the Fourth HPLC Separation Using Microbore Column

Faction[a]	Dose[b] (μg)	Activity[c]
c-1	0.1	--
c-2	0.4	--
c-3	1.3	++
c-4	0.2	--

[a]For peak assignments see Fig. 5B.
[b]Doses injected into a larva. The peptide amount was estimated by BCA protein assay with BSA as the standard.
[c]See Table 1.

TABLE 4. Summary of Comparison of ESI-MS and MALDI-TOF-MS in Application to Scorpion Toxin

	ESI-MS	MALDI-TOF-MS
Mass accuracy	1 m.u.	0.1% (varies)
Mass resolution	>1,000	<400
Sample consumption	170 pmoles	<40 pmoles
Limit of detection (3N)	170 pmoles	13 pmoles
Quantitation	Feasible	Difficult


A
Chlorotoxin: LqhIT2
8 : 67 pmol/µL
100
%
0
1126.8
966.4
667.3
799.5
996.6
845.4
572.2
740.7
1351.9
600
800
1000
1200
1400
Da/e
Raw Data
100
%
0
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3997
4000
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6000
7000
mass
MaxEnt Processed Data
B
Low Laser Power
3993.0Da
Chlorotoxin
18 pmoles
4056.5Da
LqhIT2
30 pmoles
6754.5Da
8002.7Da
10 pmoles
18 pmoles
High Laser Power
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Figure 6.

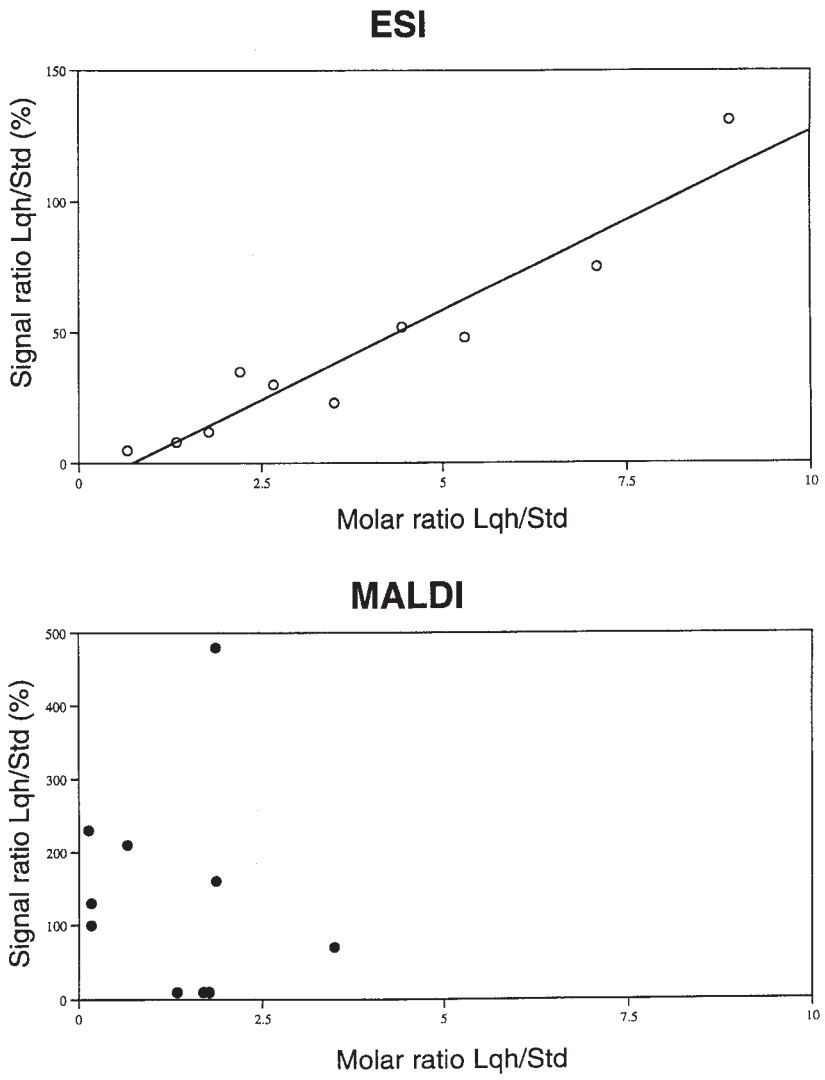

Fig. 7. Dependence of the signal ratio LqhIT2/standard (chlorotoxin) on the relative molar amount of LqhIT2 in the mixture for ESI and MALDI.

Molecular Structures of Toxins

The sequence homology of AaIT4, *Leiurus quinquestriatus hebraeus* depressant anti-insect toxin (LqhIT2), and newly identified AaIT5 toward variant 3 were found to be 44.4%, 60.0%, and 55.7%, respectively, in the Needleman and Wunsch (1970) algorithm. Five structurally conserved regions (SCR) were identified; their three-dimensional structures were built by using the coordinates of crystal structure of variant 3 (Fontecilla-Camps et al., 1980; Zhao et al., 1992) as a common template. Regions other than SCR were designated as loops assigned by loop searching routine in Homology. This algorithm searches PDB for proteins whose sequences are structurally similar to the model. Four disulfide bridges were built into the model according to their alignment to the same in the template. The model was refined by a conformational searching of mutated side chains using the Autorotamer command in Homology, in which 50 iterations of steepest descents minimization was employed at a nonbond cutoff of 8 Å. Loop splice points were repaired by 500 iterations of steepest descents and conjugate gradient minimization while applying a torsion force constant of 50 kcal/Å^2 to the peptide bond, tethering the heavy atoms of the residue in the

Fig. 6. **A:** ESI mass spectrum of chlorotoxin with LqhIT2 (80 pmoles and 670 pmoles, respectively, injected in 10 μl of 50% acetonitrile); raw data (left) and MaxEnt transformed data (right). **B:** MALDI-TOF mass spectrum of total amount of LqhIT2/chlorotoxin deposited (matrix: α-cyano-4-hydroxy cinnamic acid).

loop next to the splice point and fixing of the atoms contained in the SCR. The model was relaxed with 100 iterations of steepest descents and 200 iterations of conjugate gradients using a force constant of 100 kcal/Å^2 to tether minimizing atoms. Finally, the entire model was submitted to energy minimization of 100 and 200 iterations of steepest descents and conjugate gradients, respectively, at a constant dielectric of 30. The model was evaluated for quality by using Prostat command in Homology in which 80.3% , 72.4%, and 71.2% of the amino acids were found to exist in the core region of the psi, phi map for AaIT4, LqhIT2, and AaIT5, respectively.

The 3-D structures of AaIT5, AaIT4, and LqhIT2, as well as variant 3, are shown in ribbon form in Figure 8. The modeled 3-D structures were very similar among three insect toxins, AaIT4, AaIT5, and LqhIT2. Secondary structure such as α-helix and β-sheet are also conserved in all three toxins, only the length of α-helices varies. Recently the 3-D structure of anti-insect and anti-mammal Lqq3 in solution was determined using NMR (Landon et al., 1997). The structure of Lqq3 is topologically similar to variant 3, in which cysteine stabilized α-helix β-sheet (CSαβ) motif is recognized. The solution (Lee et al., 1994) and crystal structures of the variant 3 (Zhao et al., 1992) have similar overall features. A similar CSαβ motif is found not only in the short- and long-chain scorpion toxin (Darbon et al., 1991; Jablonsky et al., 1995), but also in plant thionins (Bruix et al., 1993). Interestingly, it is reported that Lqq3 acts on insect as the depressant toxin (Kopeyan, 1993). The specificity of toxin recognition between insects and mammals seems to be determined by very precise arrangement of the toxin and receptor.

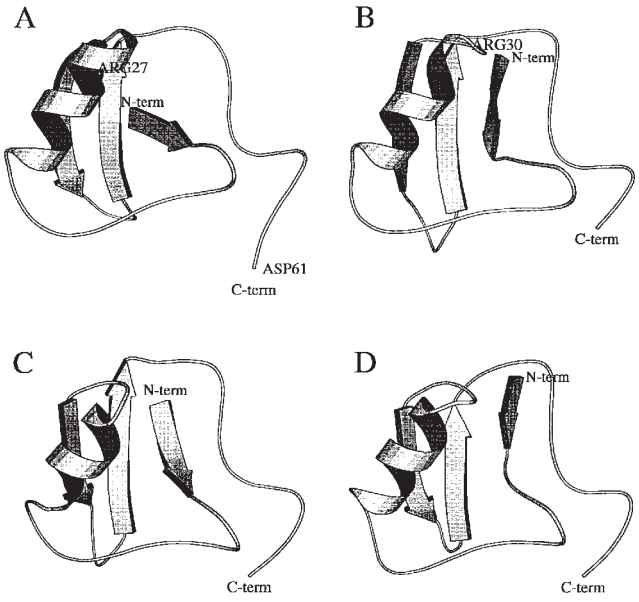

Fig. 8. Comparisons of three-dimensional structures of AaIT5 **(A)**, AaIT4 **(B)**, LqhIT2 **(C)**, and variant 3 **(D)**. The 3-D structure of variant 3 is obtained from PDB, and other structures are built from variant 3, using a molecular modeling software InsightII.

The primary structures of a number of scorpion toxins are compared in Figure 9. The amino acid sequences of all toxins obtained from *A. australis* and some other toxins from the venom of different scorpion species were aligned manually with gaps to emphasize high sequence similarity, as shown in Figure 9. Although two amino acids of AaIT5 could not be determined by direct sequencing, those are expected to be Arg27 and Asp61 based on the molecular weight information from ESI-MS and sequence homology as discussed elsewhere (Nakagawa et al., 1997). The Arg27 residue is located on the edge of α-helix in the structure in Figure 8A. Arg30 from AaIT4 (Fig. 8B) is located in similar place as Arg27 in AaIT5. As the α-amino group and Arg residues are known to play an important role in the receptor binding (Kharrat et al., 1990), these Arg residues may participate in toxin binding and give it the specificity of action.

Molecular modeling showed that AaIT5 is more similar to depressant LqhIT2 in the 3-D structure than to AaIT4. This finding is consistent with the earlier observation (Nakagawa et al., 1997) that blowfly larvae treated with AaIT5 did not contract as they did after injection of AaIT as reported.

CONCLUSIONS

In current biological and biochemical applications, MS is gaining a privileged position. We showed the fundamental application of MS to the monitoring of the purification process. Mass information can substitute the bioassay monitoring and helps to simplify, make all the purification process more accurate and time and material efficient. MS was previously used for verification of Edman sequencing (Nakagawa et al., 1997). In future work we plan a complete implementation of on line separation with MS detection, which will further increase throughput and facilitate characterization of new peptide toxins.

The improved purification method is benefi-

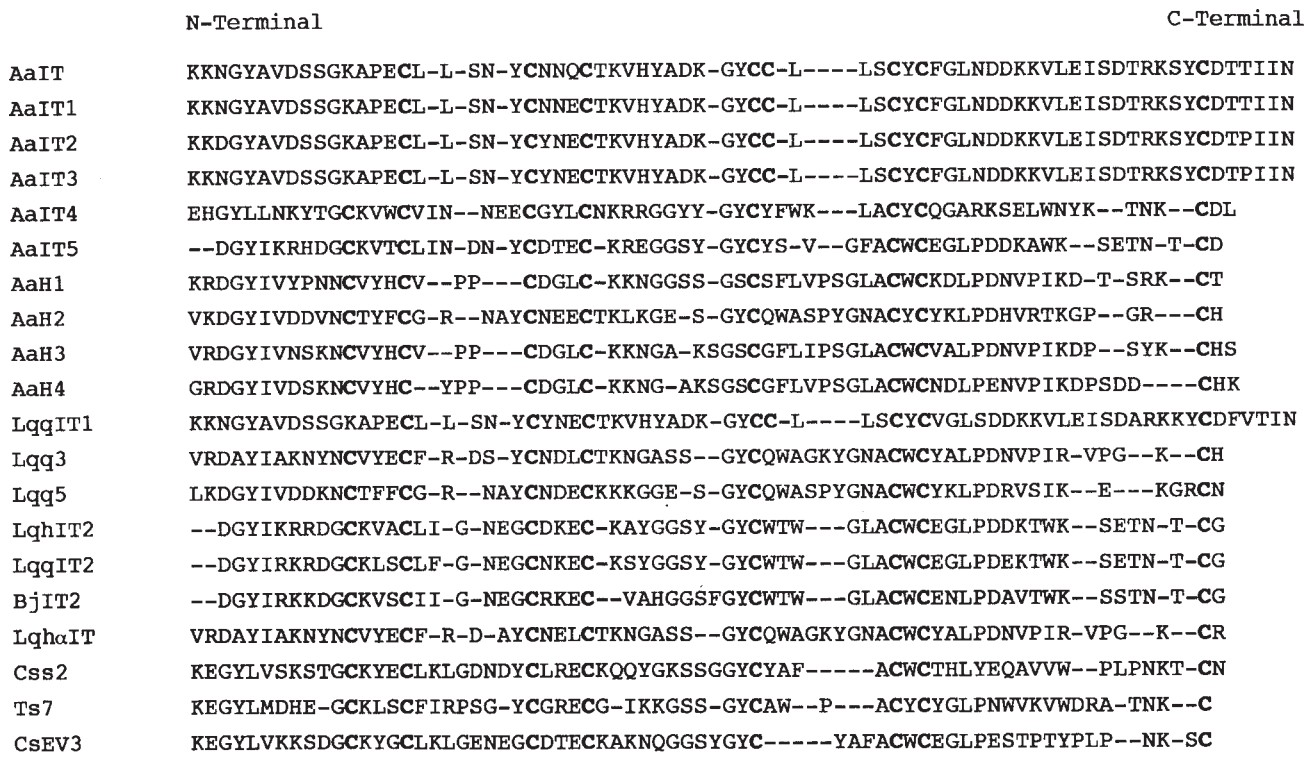

Fig. 9. Comparisons of sequences of representative insect and mammal toxins. Primary sequences were cited from the following references: AaIT (Darbon et al., 1982); AaIT1 and AaIT2 (Loret et al., 1990); AaIT3 (Bougis et al., 1989); AaIT4 (Loret et al., 1991); AaIT5 (Nakagawa et al., 1997); LqqIT1 (Kopeyan et al., 1990), LqqIT2, LqhIT2, BjIT2 (Zlotkin et al., 1991); LqhαIT (Eitan et al., 1990); AaH1 and AaH3 (Martin and Rochat, 1984; Kopeyan et al., 1979); AaH2 (Rochat et al., 1972); AaH4 (Mansuelle et al., 1992); Lqq3 (Kopeyan et al., 1993); Lqq5 (Kopeyan et al., 1978); Css2 and Ts7 (Bechis et al., 1984; Martin et al., 1987). CsEV3 (Babin et al., 1974; Fontecilla-Camps et al., 1980).

cial for the isolation of major components from the venom. A sufficient amount of AaIT to perform biological activity study was purified by only two HPLC runs from the crude venom. It was also shown in the example of AaIT5 that the new method permits isolation of enough material of minor toxin present in the crude sample to perform a bioassay and establish primary structure. This initial information can be used in transgenic expression of toxins to provide sufficient amount of material for careful pharmacology and 3-D structural analysis. Molecular modeling of rigid peptides with four intramolecular disulfide bridges as AaITs and other scorpion toxins provides the information which helps to understand the structure–function relationship.

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