Analysis of Cyclotides in *Viola ignobilis* by Nano Liquid Chromatography Fourier Transform Mass Spectrometry

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**Abstract:** Cyclotides are macrocyclic knotted peptides originating from plants. They are extremely stable and have a range of bioactivities including anti-HIV and insecticidal activity. Given the stability of the cyclotide framework, there is interest in using these peptides as scaffolds in drug design. In the current study, we have shown that nano-LC Fourier transform mass spectrometry (FTMS) is an effective method of analyzing cyclotides in plants. In addition, we have used this technique to find cyclotides in a novel species, *Viola ignobilis* (Violaceae plant family), which was collected from the East Azerbaijan province of Iran. Varp peptide A, cycloviolacin B2, and cycloviolacin O8 were found in this species. This study provides a novel method for directly analyzing cyclotide sequences without enzymatic digestion and further information regarding the distribution of cyclotides in plant species.

**Keywords:** Cyclic peptide, Cyclotide, nano-LC FTMS, Varp peptide A, *Viola ignobilis*.

**INTRODUCTION**

Cyclotides are macrocyclic plant peptides with three disulfide bonds and a head-to-tail cyclized backbone. These topological features provide the cyclotides with a unique structure, and remarkable stability [1]. Cyclotides typically comprise 28-37 amino acids and are the largest known family of circular proteins [2]. They are resistant to enzymatic degradation, and stable at high temperature and under a range of pH conditions [3]. The natural function of cyclotides is thought to be in host defense against a range of pests and pathogens [4]. The first cyclotide, kalata B1, was isolated from a decoction made from boiling *Oldenlandia affinis* leaves and displayed uterotonic activity [5]. Since this discovery a wide variety of biological activities for cyclotides have been reported including cytotoxic [6], antimicrobial [7], anti-HIV [8] and haemolytic activity [9].

Mass spectrometry is a conventional and powerful technique for phytochemical analyses [10, 11]. Indeed, determination of the primary structure of cyclotides has been carried out by tandem mass spectrometry techniques. Using this approach cyclotides have been isolated from crude extract and after enzymatic digestion, MS/MS carried out [12]. Matrix assisted laser ionization/desorption time-of-flight mass spectrometry (MALDI-TOF MS) has been used to directly analyse cyclotides based on molecular weight and MS/MS fragmentation patterns [13-15].

The matrix of cyclotides in plants is complex and although primary sample preparations have been introduced before chromatographic or electrophoresis analysis, good resolution for cyclotides by electrophoresis and HPLC can be difficult [13]. Combining nano-LC with FTMS (nano-LC FTMS) has significant advantages in terms of resolution of components and provides a unique identification technique for natural compounds [16-18]. In addition FTMS has high accuracy and is capable of providing monoisotopic masses to five decimal places. Therefore, known compounds can potentially be indentified by simply comparing theoretical and experimental monoisotopic masses [19].

Peptide sequence analysis by mass spectrometry has been used in top down or bottom up strategies. Bottom up peptide sequencing is performed using enzymes for cutting peptides into smaller fragments but in top down methods enzymes are not required. Consequently, the top down strategy is not a cost or time consuming method [20]. In this work we applied a top down peptide sequencing strategy for identification of cyclotides in *Viola ignobilis* from a crude extract. To this end we applied a simple clean up process prior to direct measurement by mass spectrometry. To the best of our knowledge this work is the first report on of the analysis of cyclotides by nano-LC/ FTMS.

**MATERIALS AND METHODS**

**Extraction and Isolation of Cyclotides From Viola ignobilis**

Plant material form *Viola ignobilis* was collected in spring 2008 from Arasbaran of East Azerbaijan in Iran. Plant material (~140 g) was ground and extracted with dichloromethane/methanol (1:1, v/v) overnight. The sample extract was then partitioned with dichloromethane and methanol by introducing water and the aqueous phase was concentrated...
prior to freeze drying. The sample was dissolved in ammonium bicarbonate buffer (pH 8) and passed through C18 SPE columns. During this process the polar compounds passed through the column but other compounds absorbed onto the C18 sorbents. Compounds were eluted with 20%, 50% and 80% ethanol. These eluted fractions were freeze-dried and concentrated in preparation for RP-HPLC and nano-LC FTMS and other analyses.

**NANO-LC FTMS ANALYSIS**

For nano-LC FTMS analysis, the Dionex/LC Packings (Idstein, Germany) Ultimate binary nano HPLC pump/autosampler system was used. A trap column (Dionex, C18 PepMap, I.D. 300 μm, length 5 mm) for sample injection was applied with 1 μL of a 1:10 dilution of the sample pre-focused on it. Then this sample was separated on a fused-silica C18 PepMap 100 capillary column (Dionex, 3 μm, 100 Å; I.D. 75 μm; length 150 mm). HPLC analysis performed by using a linear gradient from 25% solvent B (0.08% FA in 80% ACN) to 45% over 50 min at a flow rate of 0.030 ml/min. Solvent B was 0.01% FA in 2% ACN.

A Finnigan LTQ FT Ultra hybrid instrument (Thermo Fisher Scientific, Bremen, Germany) with a linear quadropole ion trap and a Fourier transform ion cyclotron resonance mass spectrometer with a 6 Tesla magnet was used to acquire mass spectra. A nanospray ionization device was applied for sample analyses (spray voltage 1.0-2.0 kV, capillary temperature 250°C, capillary voltage 10 V, tube lens 100 V). Mass resolution was set to 100,000. Mass accuracies were found to be better than 1 ppm in most cases.

**RESULTS AND DISCUSSION**

The sample preparation of cyclotides prior to HPLC analysis is very important to allow suitable separation of components. Several reports have used different extraction methods for cyclotides [14]. In this work, SPE was selected for isolation of cyclotides from the complex media of *V. ignobilis*. Therefore, a fraction containing cyclotides was obtained from *V. ignobilis* after the plant extract was passed through SPE. Ethanol was used as the elution solvent in SPE at 20%, 50% and 80%. The highest extraction yields were obtained with 50% ethanol.

Although several studies on the isolation and characterisation of cyclotides in plants using conventional LC-MS (liquid chromatography-mass spectrometry) systems have been reported, this is the first report of using nano-LC FTMS in the analysis of cyclotides. A high resolution chromatogram for cyclotides of *V. ignobilis* was obtained with nano-LC as shown in Fig. 1. The separation was optimised using several different gradients.

Although two purification steps, solvent extraction and SPE, were used for isolating the cyclotides, it is clear from Fig. 1 that these methods were not sufficient to remove all non-cyclotide components. The results showed that three masses at 12.70, 12.85, and 23.44 min of TIC (total ion current) are in the range of cyclotide masses [18]. These three cyclotide masses are in the Cybase database [21, 22] but there are three cyclotides with same mass for 23.44 min peak. The TIC and mass spectra of the three cyclotides are shown in Fig. 2. The ions have three positive ions and the monoisotopic masses of these compounds were compared with theoretical weights of known cyclotides in Table 1. The three masses at 3166.402, 3225.425 and 2876.127 m/z have been identified that are similar to known cyclotide masses with errors less than 1 ppm. The first two peaks correspond to the masses of cycloviolacin B2 and cycloviolacin O8. However, the third peak corresponds to Varv peptide A, C and D.

![Figure 1. nano-LC-MS for the extract of *Viola ignobilis*. The extract was partially purified using solid phase extraction prior to analysis with nano-LC. The trace highlights the retention times of the three cyclotides and their relative intensity. The conditions used in the nano-LC are outlined in the Methods section.](image-url)
Figure 2. Total ion currents and mass spectra of cyclotides from *Viola ignobilis*. The traces are for cycloviolacin O8 (A), cycloviolacin B2 (B) and varv peptide A (C).
Table 1. Comparison between Experimental and Theoretical Masses for Selected Cyclotides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Experimental Mass</th>
<th>Theoretical Mass</th>
<th>^{\text{Difference} \times 10^6}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloviolacin B2</td>
<td>3166.39977</td>
<td>3166.39065</td>
<td>2.8</td>
</tr>
<tr>
<td>Cycloviolacin O8</td>
<td>3225.42516</td>
<td>3225.42494</td>
<td>0.06</td>
</tr>
<tr>
<td>Varv peptide A</td>
<td>2876.12736</td>
<td>2876.12692</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*The difference between the experimental and theoretical is expressed as a fraction of the experimental mass.

Figure 3. Total ion currents and mass spectra of cyclotides from *Viola ignobilis* following reduction and alkylation of the disulfide bonds. The traces are for cycloviolacin O8 (A), cycloviolacin B2 (B) and varv peptide A (C). The peptides were reduced with dithiothreitol and alkylated with iodoacetamide.
Table 2. Comparison of Experimental and Theoretical Masses for Reduced and Alykated Cyclotides

<table>
<thead>
<tr>
<th>Cyclotide</th>
<th>Experimental Mass</th>
<th>Theoretical Mass</th>
<th>Difference (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloviolacin B2</td>
<td>3514.574793</td>
<td>3514.59528</td>
<td>5.8</td>
</tr>
<tr>
<td>Cycloviolacin O8</td>
<td>3573.600678</td>
<td>3573.62316</td>
<td>6.2</td>
</tr>
<tr>
<td>Varv Peptide A</td>
<td>3224.302648</td>
<td>3224.45763</td>
<td>48</td>
</tr>
</tbody>
</table>

Figure 4. MS/MS spectra of an ion from varv peptide A. The triply charged 1076 ion was subjected to MS/MS and the data used to derive various fragments of varv peptide A which are labeled with the one-letter code.

Figure 5. The sequences of three candidate cyclotides for the fraction with a mass at 2876.12736. Two fragments that were used to distinguish between the three peptides are highlighted in bold. The cyclic backbone is indicated with a line connecting the N- and C-termini.

Figure 6. A summary of the fragments used to determine the sequence of varv peptide A. The fragments are connected by lines with the masses for individual fragments highlighted.
To further characterize the peptides, the disulfide bonds were reduced with dithiothreitol, alkylated with iodoacetamide, the reagents then removed and the sample used for sequencing studies. Enzymatic digestion was not required as has been commonly used in previous studies on cyclotide sequencing. The reduced and iodoacetamidated cyclotides showed mass increases of 348.174 Da. The calculated reduced masses for the three cyclotides are given Table 2. The TIC spectra of the reduced cyclotides are shown in Fig. 3. The differences between the theoretical and experimental values (Table 2) are greater than those observed for the native peptides. This phenomenon might be related to the low yields obtained after reduction and alkylation of the peptides resulting in higher errors in the measurements.

To identify if the peak with the mass of 2876.12736 was one of the three candidates (Varv peptide A, Varv peptide C, Varv peptide D), the triple positive ion 1076 was subjected to MS 2 . The mass spectra of 1076 is shown in Fig. 4. The sequences of the three candidate cyclotides with a mass of 2876.12736 are shown in Fig. 5. Two fragments of 591 and 702 m/z were observed in MS 2 of 1076. Varv peptide A and C both have a fragment of 591 m/z related to the GGTTCNT amino acid sequence. Varv peptide D does not have this sequence and therefore was ignored as a candidate for this peptide. Given that the sequence CTRNGL has a m/z of 702 and is solely present in Varv peptide A, varv peptide C could also be discarded. Consequently, Varv peptide A has been identified as 2876.127 eluting at 23.34 min. Mass fragments of Varv peptide A are shown in Fig. 6.

The peptides corresponding to 3166.402, 3225.425 were also be discarded. Consequently, Varv peptide A has been solely present in Varv peptide A, varv peptide C could both have a fragment of 591 m/z related to the GGTCNT sequence and structure of antimicrobial peptides containing end-to-end macrocycle and cystine-knot disulfides. Proc. Natl. Acad. Sci. U.S.A. 1999, 96, 8913-8918.

REFERENCES