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Microwave-assisted extraction of cyclotides from *Viola ignobilis*

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ABSTRACT

Cyclotides are an interesting family of circular plant peptides. Their unique three-dimensional structure, comprising a head-to-tail circular backbone chain and three disulfide bonds, confers them stability against thermal, chemical, and enzymatic degradation. Their unique stability under extreme conditions creates an idea about the possibility of using harsh extraction methods such as microwave-assisted extraction (MAE) without affecting their structures. MAE has been introduced as a potent extraction method for extraction of natural compounds, but it is seldom used for peptide and protein extraction. In this work, microwave irradiation was applied to the extraction of cyclotides. The procedure was performed in various steps using a microwave instrument under different conditions. High-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) results show stability of cyclotide structures on microwave radiation. The influential parameters, including time, temperature, and the ratio of solvents that are affecting the MAE potency, were optimized. Optimal conditions were obtained at 20 min of irradiation time, 1200 W of system power in 60 °C, and methanol/water at the ratio of 90:10 (v/v) as solvent. The comparison of MAE results with maceration extraction shows that there are similarities between cyclotide sequences and extraction yields.

Cyclotides are a large family of miniproteins that have been isolated from plants of the Rubiaceae, Violaceae, Cucurbitaceae, Fabaceae, Solanaceae, and Poaceae families [1–7]. Cyclotides are between 27 and 37 amino acids in size. They are head-to-tail macrocyclic peptides containing six conserved cysteine residues that form three disulfide bounds in a cystine-knotted arrangement [8,9]. This interesting three-dimensional structure confers them exceptional stability against chemical, enzymatic, and thermal treatment, which would typically degrade linear proteins of similar size. Due to their stability, cyclotides have been considered as a promising template for peptide engineering and pharmaceutical applications [10]. In addition, they exhibit intrinsic biological activities such as uterotonic [4,5,11], anti-HIV [12–14], antitumor [15], anthelmintic [16], insecticidal [17,18], cytotoxicity [19,20], and immunosuppressive properties [6,21,22]. Cyclotides are interesting compounds for using in drug design. Pharmaceutical application of them is related to their use in drug design as a template for grafting unstable peptides' sequences to give them exceptional stability [23,24].

Similar to all plant-derived natural compounds, extraction is the critical step in the isolation of cyclotides from plant tissue. In previous studies, methanol and dichloromethane [25,26], or a combination of water and alcohols [27], have been used most commonly as solvents for extraction of cyclotides using maceration. This method usually endures over 24 h to obtain efficient extraction yields. Hence, other methods that could reduce extraction time are needed.

Recently, the microwave-assisted extraction (MAE) technique has become attractive for the extraction of thermo labile components because of its properties such as little solvent consumption and fast extraction efficiency with high yield [28,29]. Microwave

Abbreviations: MAE, microwave-assisted extraction; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; UV–VIS, ultraviolet–visible; ESI–MS, electrospray ionization mass spectrometry; MALDI–TOF/TOF, matrix-assisted laser desorption/ionization tandem time-of-flight; LC, liquid chromatography.

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radiation has been shown to increase the temperature of aqueous solutions by dielectric heating [30]. In the case of proteins, prolonged irradiation using microwaves has also been proposed to cause protein unfolding and disruption of tertiary structure and enzyme activity [31]. For instance, Guan and coworkers reported on the effect of microwaves on isolation of soy protein, revealing that under a long time exposure to high-power microwave irradiation, noncovalent bonds and the disulfide bonds in soybean protein were broken. But protein was disaggregated and unfolded, suggesting that forces that handle protein structures, such as hydrophobic forces, can be destroyed by microwaves to a certain extent as a result, but less time will be spent on the microwave-assisted soy protein–saccharide graft reactions; therefore, higher yields and fewer by-products would be obtained [32]. However, Shin and coworkers observed no significant structural changes of human hair proteins using microwaves (600 W) for a specified irradiation period (5–120 min) due to the high cysteine content and, hence, great stability of hair proteins [33]. In many cases, direct interaction of protein or enzyme structure with the electromagnetic field of the microwave reactor was attributed to nonthermal effects [34–36], and evaluation of tertiary structure of trypsin and bovine serum albumin by molecular mechanics calculations revealed that the employed electromagnetic field strength under laboratory microwave conditions without any significant heating is too low to induce conformational changes in proteins or enzymes [37,38].

Because cyclotides are ultrastable miniproteins, the aim of this work was to evaluate the effectiveness of cyclotide extraction from *Viola ignobilis* using microwave irradiation and to determine overall yields of extracted cyclotides due to electromagnetic field effects.

1. Materials and methods

1.1. Plant collection and extraction

At first, the dried plant material of *V. ignobilis* (100 g) that was collected from the region of East Azarbaijan, Iran, was powdered. Then, the dichloromethane extraction at room temperature for 24 h as an appropriate extraction process was used to remove the nonpolar components. After filtering through a cotton wool plug, the pulp plant was collected, dried, and prepared for cyclotide extraction by maceration and microwave extraction methods using polar solvents.

1.2. Microwave extraction

MAE was performed using a Multiwave 3000 (Anton Paar, Graz, Austria) equipped with 16 closed vessels; the maximum operating temperature and pressure were 190 °C and 20 bars, respectively. For this method, 1 g of the powdered plant material was weighed directly into each pre-cleaned microwave vessel and 30 ml of different ratios of methanol/water was added to each vessel. Following incubating periods of 4, 8, 12, 16, 20, and 24 min with different conditions (Table 1), the mixture was cooled and the solvent was separated from the solid material using a separating funnel. The resulting solutions were concentrated on a rotary evaporator and freeze-dried; the crude extract was dissolved in 0.1 M NH_4HCO_3 buffer (pH 8.1) and used for solid-phase extraction (SPE) of cyclotides. For comparison of yields of this method with maceration, conventional maceration was performed under optimal conditions; that is, 30 ml of different ratios of methanol/water was used as extraction solvents for 1 g of plant material over 24 h, similar to the method described by Yeshak and coworkers for extraction of cyclotides from *Viola odorata* [27].

1.3. Solid phase extraction and chromatography analysis

C_{18} SPE cartridges (0.5 g, 3 ml; Macherey–Nagel, Chromabond) were activated with 3 ml of MeOH and equilibrated with 3 ml of water. The dried crude extract, dissolved in 0.1 M NH_4HCO_3 buffer, was loaded onto the C_{18} cartridge and washed with 10 ml of 0.1 M NH_4HCO_3 buffer, followed by 10 ml of 20% ethanol. The cyclotide-containing fractions were collected by elution with 50 and 80% ethanol, respectively, and freeze-dried. After dissolving in 5% acetonitrile, they were analyzed using RP-C_8 high-performance liquid chromatography (HPLC) columns (250 × 4.6 mm, 5 μm, 100 Å; Eurospher I, Knauer), using a Knauer 1200 series unit, with the following mobile phases: solvent A (100% water containing 0.05% trifluoroacetic acid [TFA]) and solvent B (water/acetonitrile [10:90, v/v] containing 0.05% TFA). A flow rate of 1 ml/min was used for the analysis. The gradient employed started at 95% A and remained at this point for 5 min before changing to 85% over 10 min. A linear gradient starting from 85 to 0% A was then employed during a 25-min period, remaining at 100% B for a further 5 min. Afterward, the column was reequilibrated to 95% A in 10 min. Peptide elution was monitored by ultraviolet–visible (UV–VIS) detection at 218 nm.

1.4. Mass spectrometry analysis

To investigate effects of microwave irradiation on the structure of the cyclotides, extracted samples were analyzed by a Thermo-Fisher Scientific electrospray ionization mass spectrometry (ESI–MS) system equipped with an ion trap mass spectrometer (model LCQ, mass range *m*/*z* 10–2000) and a nanospray ionization interface (Bremen, Germany). Instrument control and data acquisition and processing were conducted by Xcalibur software. Typical positive ESI–MS conditions were as follows: capillary voltage 4.0 kV and skimmer cone voltage 20 V. Matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI–TOF/TOF) was performed using a 4800 Analyzer (AB Sciex, Canada) operated in reflector positive ion mode, acquiring 2000 to 3600 total shots per spectrum with a laser intensity set between 3200 and 3800. MS experiments were carried out using α-cyano-hydroxylcinnamic acid matrix at a concentration of 5 mg/ml dissolved in 50% (v/v) acetonitrile. An aliquot of 0.5 μl of each sample was mixed with 3 μl of matrix solution, and the mixture was spotted onto the target plate [39,40].

Table 1
Microwave extraction process.

Microwave extraction process	Temperature (°C)	Solvent (% methanol)	Irradiation time (min)	Microwave power (W)
Step 1	50	70, 80, 90	4	500
Step 2	60	90	4	500
Step 3	60	90	4	1200
Step 4	60	90	8, 10,12, 16, 20	1200