Talanta 131 (2015) 640–644

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Peptidomics study of anthocyanin-rich juice of elderberry

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ARTICLE INFO

Article history:
Received 13 June 2014
Received in revised form
3 August 2014
Accepted 6 August 2014
Available online 27 August 2014

Keywords:
Elderberry
Peptidomics
Solid phase extraction
Orbitrap-MS/MS
Anthocyanin *sambucus*

ABSTRACT

Biologically active peptides play a role in plant signaling and defense. Elderberry juice is known to contain a variety of anthocyanin compounds, a sub-set of polyphenols, which are responsible for the deep purple color of the juice. In this paper, we describe a method utilizing solid phase extraction (SPE) to remove anthocyanins from peptides. Liquid chromatography coupled with tandem mass spectrometry was used to separate and identify the peptides. The results showed that the use of SPE was an effective method to separate peptides from anthocyanins and other background compounds including high polyphenol content in the juice samples. More than 1000 peptides present in elderberry juice were successfully identified.

1. Introduction

Thousands of peptides have been isolated and purified from microorganisms, animals, and plants. Plant peptides are often key players in cell-to-cell communication governing plant development. They also play a role in communication between plants and other organisms. Plants are an enormously rich source of peptides, with the potential to be developed as nutrients, medicines and biomarkers [1,2]. Some secreted peptides are recognized as important hormones that coordinate and specify cellular functions in plants. Peptides are of interest as they possess several advantages including low molecular weight, relatively simple structure, ease of absorption, and lower antigenicity [3–5]. The first functional plant peptide to be identified was tomato systemin, an 18 amino acid polypeptide, which acts in the rapid expression of defense-responsive genes via cellular communication [6]. In the 1990s, a disulfated pentapeptide was isolated by Matsubayashi and Sakagami, and named as phytosulfokine. It is a potent mitogenic factor from conditioned medium derived from cultures of asparagus mesophyll cells [7]. Studies over the past decade have highlighted the diverse nature of peptides and explored their possible uses.

Peptidomics is a fast, efficient method that can detect low and transient concentrations of peptides and identify their post-translational modifications. It is a branch of proteomics that has been developing over several years. There are several approaches to peptide separation and identification [8]. Two-dimensional gel electrophoresis can be extended to detect smaller peptides (1 kDa and larger) by the use of tris-tricine in the second dimension. After extraction from the gel, the isolated peptides can be measured by MALDI-TOF-MS. The combination of high performance liquid chromatography (HPLC) coupled with MS/MS can help to overcome complex peptide mixtures and aid in the identification of less abundant peptides. The use of nano-liquid chromatography can increase the signal-to-noise ratio and sensitivity of MS for the detection of peptides in complex mixtures or those present in low concentrations [9–12].

Peptidomics studies the role of peptides and bridges the space between proteomics and metabolomics [13]. The full spectrum of peptide functions in plants is unknown. Peptide hormone signaling in plants is an emerging area of research and peptides have been shown to affect cell division, development, reproduction, nodulation and defense. The major difference between proteomics and peptidomics, regardless of peptide size, is that peptidomics identifies native peptides. Thus, it can be referred to as a version of top-down proteomics which considers only the peptidome (peptides).

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http://dx.doi.org/10.1016/j.talanta.2014.08.022
0039-9140/

Elderberry (*Sambucus* spp) has been used as a traditional medicine and its use can be traced back to the Roman times. The juice is known to be rich in anthocyanin compounds and can contain peptides. Elderberry juices have been to vary with cultivar and growing regions and conditions [14,15]. Till date, no peptide profiling of elderberry has been reported [16–20]. In this paper, we describe a method to remove the anthocyanin from the peptides in elderberry juice by solid phase extraction and use LC-MS/MS to profile and *de novo* sequence the peptides. Analysis of the peptide sequences was then used to identify possible proteins present in the juice samples.

2. Materials and methods

2.1. Materials and instruments

Methanol, formic acid, ammonium hydroxide and other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA). American elderberry juice (*Sambucus nigra* L. subsp. *canadensis* (L.) Bolli; syn. *S. canadensis* L.) was prepared from fruit grown in a commercial orchard in central Missouri, USA. Berries from cultivar 'Wyldewood' were harvested at peak ripeness in early September 2011, destemmed, pressed, and the juice frozen. The juice was later thawed and aliquoted (pH 4.51) and then filtered through a 0.45 μm nylon filter to remove any suspended solids. The color of the juice was dark-violet. Solid phase extraction (SPE) cartridges (Oasis MCX (3cc, 60 mg) and C_{18} Certified Sep-Pak Vac (3cc, 500 mg)), were purchased from Waters Corp. (Milford, MA). MALDI spectra were acquired on an Applied Biosystems 4700. LC-MS/MS spectra were acquired on a LTQ Orbitrap XL mass spectrometer (Thermo Scientific) with an interfaced nano-LC (Proxean Easy nLC-1000).

2.2. Peptide separation and purification

The Oasis MCX stationary phase contains two types of materials: a strong cation-exchange phase with a C_{18}-reversed phase. Two cartridge volumes of elderberry juice in aqueous solution (12 mL) were applied to an MCX SPE cartridge (6 mL, 1 g sorbent; Waters Corp., Milford, MA). After washing with 12 mL of water (0.1% FA), the peptide fraction was collected by elution with 12 mL of methanol (0.1% FA) retaining the anthocyanins on the column. The anthocyanin fraction was then eluted with 6 mL of methanol and 6 mL of water:methanol (40:60, v/v), both containing 1% NH_4OH. The combined alkaline eluate was immediately mixed with an aliquot (250 μL) of formic acid (99%) to lower the pH to less than 2 to prevent degradation of the anthocyanin fraction.

The peptide fraction was briefly evaporated in a rotovaporator at 90 °C for 10 min to remove the organic solvent, redissolved in water (0.1% FA), filtered through 0.45 μm polypropylene filter, and then separated into two groups for analysis. One group was analyzed by MS directly (Group A) and the other group was further purified using a C_{18} SPE cartridge (Group B) following the protocol provided by the manufacturer. Fig. 1 is an overall flow chart of the procedure used for the separation of the peptides from the elderberry juice.

2.3. MALDI-TOF MS/MS detection

Elderberry juice samples were tested by MALDI-TOF MS/MS. Each of the samples was combined 1:1 (v/v) with α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), or sinapinic acid (SA) matrix for application to the target for MALDI-TOF MS analysis. MS spectra were acquired in the positive ion reflector delayed-extraction mode with external calibration.

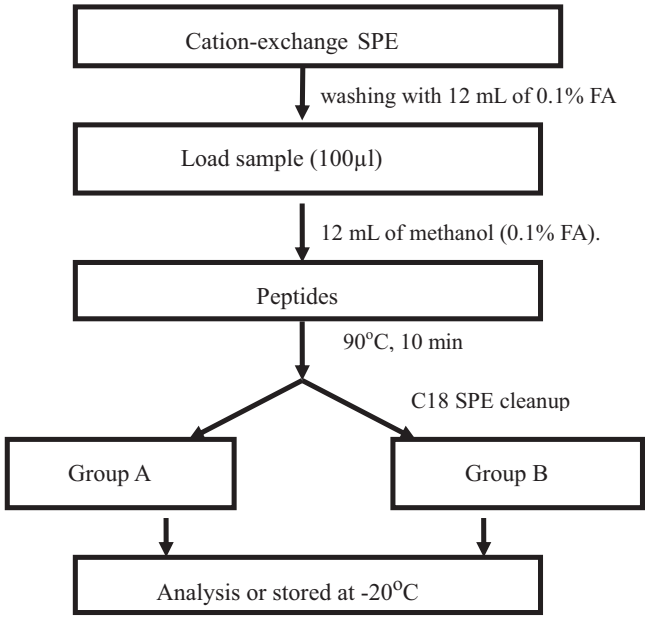

Fig. 1. Experimental scheme for the purification and isolation of peptides in elderberry juice.

2.4. Peptide identification

Data was acquired on an LTQ Orbitrap XL with an interfaced nano-HPLC system. Nano-liquid chromatography conditions are described below. Mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in acetonitrile, flow rate 20 μL/min, sample loading volume 30 μL (flow rate 25 μL/min), max pressure 200.00 bar. The gradient program is shown as Supplementary Table S1 with a total of 120 min running time. The following LC-MS parameters were applied. Scan event 1: analyzer FTMS, mass range normal, resolution 30,000, full-scan, polarity positive, data type profile; scan event 2: analyzer ion trap, mass range normal, scan rate normal, data type centroid.

The peptide mixture was loaded onto a trap column (C_{18}, 75 μm × 100 mm) and separated on a MonoSpray C_{18} tip using a 120 min gradient from 10 to 50% acetonitrile in 0.5% formic acid at a flow rate of 200 nL/min. The protonated ion of polycyclodimethylsiloxane (445.120025 Da) was used for internal calibration throughout. The mass spectrometer alternated between a full FTMS scan (300–1500 Da) and subsequent MS/MS scans. Cations were isolated with an isolation window of 5 Da and provided a dynamic exclusion list for 2400 s after selected for at least two MS/MS scans. Singly charged precursors were excluded. Monoisotopic selection was disabled with an exclusion window setting of 1 Da. The four most intense ions were chosen for CID fragmentation. Automatic gain control was used to accumulate sufficient fragment ions (MS/MS target value 2E5; maximum injection time, 1000 ms) [9]. A sample blank was run before the analytical sample. The blank solution included 5% ACN and 1% FA. Peptide *de novo* sequencing was performed using the proteomics software PEAKS. A trial version of PEAKS 6 was downloaded from the bioinformatics solutions website (http://www.bioinfor.com).

2.5. Protein sequence similarity searches

BLAST protein sequence searches were performed using the measured peptide sequences (http://www.uniprot.org/blast). The parameter settings were as follows:

Program blastp (BLASTP 2.2.28+), Database: uniprotkb_viridiplantae (Protein) generated for BLAST, Sequences: 1,711,995 sequences consisting of 584,837,709 letters, Matrix: blosum62, Threshold: 0.1, Filtering: none, Gapped: yes.